TUDOR5-LIKE LOCALIZES TO A NOVEL GERMLINE CYTOPLAMIC RNA GRANULE IMPORTANT FOR POST-TRANSCRIPTIONAL GENE REGULATION IN *DROSOPHILA MELANOGASTER*

By,

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Abstract

One family of proteins that is known to play a vital role in germline development are the tudor-domain-containing proteins. Previous work in our lab identified a novel tudor-domain protein in *Drosophila* named Tudor5-like (Tdrd5l) that was capable of promoting male identity in the germline. In this dissertation I focused on understanding how Tdrd5l functions in the germline, and I identified an unexpected role for Tdrd5l in female germline development.

Most Tudor-domain-containing proteins function in an RNA metabolism pathway. In the germline many of these tudor proteins function in piRNA biogenesis and localize to an RNA granule called the nuage. Here we found that Tdrd5l localizes to a potentially novel RNA granule, unlike its closest homologues which localize to the nuage and repress transposons. The Tdrd5l granule we observe associates with the nuage and processing body but doesn't perfectly co-localize. Additionally, Tdrd5l granules are much larger than most characterized RNA granules. Lastly, we found that like many tudor proteins, Tdrd5l functions in post-transcriptional gene regulation based on genetic interaction assays.

In the female germline we found that Tdrd5l regulates a special type of RNA called maternally deposited RNAs. In this study we specifically focus on the role Tdrd5l plays in regulating the maternally deposited RNA *gurken (grk)*. My results showed that Grk protein expression is de-repressed in the nurse cells where it is usually silenced. In addition, we observed dorsalized eggs laid by *Tdrd5l* mutant females, a phenotype that

also indicates a defect in *grk* regulation. To further understand how Tdrd5l regulates Grk protein expression, we tested whether Tdrd5l regulates Orb, which is an activator of Grk translation. We found that Tdrd5l acts to repress Orb in the nurse cells, a mechanism that could explain why Grk is ectopically expressed in Tdrd5l mutants.

Overall my data demonstrate that Tdrd5l localizes to a novel RNA granule and that this protein is capable of regulating mRNAs in the female germline.

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Dedication

This thesis has two dedications:

First, I dedicate this thesis to my parents Lynn and Murray Pozmanter for all their love and support over the years, especially while I completed my doctorate

Second, I dedicate this thesis to all the wonderful students I have taught as an adjunct lecturer. They have shown me my love of teaching that provided my drive to complete my PhD.

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Chapter 1: Introduction

Gametogenesis

All sexually reproducing animals rely on a special set of cells found in the gonad called the germline to pass on their genetic information to the next generation. The germline is unique in that it is capable of producing an entire organism. Additionally, the type of gamete produced by the germline is determined by the sex of the animal and sex of the germline cells themselves. Across the animal kingdom, there are many common themes and mechanisms utilized by the germline to produce mature gametes. When these processes go wrong, the organism is rendered infertile, thus making our understanding of germ-cell biology of great importance.

Sex determination in the gonad

Whether an animal's germline gives rise to sperm or eggs is dependent on the sex of the animal. In males the testes give rise to sperm, and in females the ovaries give rise to eggs. In both mammals and flies, the sex of the germ cells is determined cell autonomously in addition to receiving instructions from the surrounding somatic cells of the gonad. Both species make their male-vs-female decision based on X chromosome composition where a cell with 2 X chromosomes is female in identity, and a cell with an X and a Y is male in identity. However, the way the cells use this information differs between flies and mammals. In mammals it is the presence of the *SRY* gene on the Y chromosome that determines male fate (Wilhelm, Palmer, and Koopman 2007). In flies it's the presence of 2 X chromosomes relative to two sets of autosomes that determine female fate (Erickson and Quintero 2007). The X chromosome gene *Sex lethal* (*Sxl*) is known as the master regulator of female sex in flies (Salz et al. 1989).

SRY and Sxl both function by triggering other genes to bring about sex-specific development (Marín and Baker 1998). It should also be noted that not every gene required for sex-specific development resides on the X and Y chromosomes (Coschigano and Wensink 1993); thus infertility disorders could arise from gene mutations on virtually any chromosome. Once a germline stem cell determines its sex it can start the developmental program of spermatogenesis to become a mature sperm cell or oogenesis to become a mature egg cell. Sperm and egg development share many of the same mechanisms in flies in addition to gamete-specific processes such as spermiogenesis and maternal RNA deposition.

Sxl and its target genes in the germline

In *Drosophila* Sxl is only expressed in cells with 2 X chromosomes and triggers the female developmental program. Sxl is an RNA binding protein capable of controlling alternative splicing of pre-mRNAs and binding to mature mRNAs to silence their translation (Li et al. 2013; Valcárcel et al. 1993). Characterized targets of Sxl such as *PHD finger protein 7* (*Phf7)* promote male identity in the germline(Yang, Baxter, and Van Doren 2012, 7). Sxl represses *Phf7* in females, while the absence of Sxl in the male germline allows for the expression of *Phf7* and a more recently identified Sxl target, *Tudor5-like (Tdrd5l)*. The regulatory logic for *Tdrd5l* by Sxl is similar to how *Phf7* is regulated. In females Sxl binds to the *Tdrd5l* transcript to repress expression in the germline stem cells, while in male germline stem cells, the absence of Sxl allows for the expression of Tdrd5l (Primus et al. 2019).

Sxl and its target genes in the soma

In addition to the germline, Sxl also promotes female fate in the soma although via a different gene regulatory cascade. In the somatic cells, default spicing of RNAs encoding two transcription factors, Doublesex (Dsx) and Fruitless (Fru), leads to the production of the male promoting isoforms of these proteins. In the presence of Sxl, *transformer (tra)* is alternatively spliced to produce a female specific isoform (Valcárcel et al. 1993), which then leads to the alternative splicing of *dsx* and *fru* into their female-promoting isoforms (Hoshijima et al. 1991; Heinrichs, Ryner, and Baker 1998). Additionally, recent data from our lab has shown that Dsx can also regulate *fru* independent of Tra. Importantly, while it's well understood how this development occurs in the soma, less is known in the germline, and Tra, Dsx, and Fru expression are restricted to the soma (Whitworth, Jimenez, and Doren 2012).

Drosophila gametogenesis

In flies, each female has two ovaries, each comprised of 16-18 ovarioles, which are chains of developing egg chambers (Fig. 1.1) (Duhart, Parsons, and Raftery 2017), while each male has two testes (Fig. 1.2). Gametogenesis in flies starts off using a similar mechanism between the male and female germlines. In both the ovary and the testis, germline stem cells (GSCs) adhere to the stem cell niche. Important components of the niche consist of the terminal filament and cap cells in females, and the hub cells in males. One difference between males and females is the number of GSCs that adhere to the niche. In the ovary each ovariole contains 2-3 GSCs adhered to the niche at the anterior end of the ovariole, while in males, 10 GSCs adhere to the hub in each testis. While there are far fewer GSCs per niche in the ovary, when you add up the number of

ovarioles per ovary, the total number of GSCs per ovary is greater than the number of GSCs per testis.

In the female germline when a GSC divides, one daughter cell remains at the niche while the other becomes a cystoblast (CB). A key protein in promoting development of the CB is an RNA binding protein called Bag of Marbles (Bam) (McKearin and Spradling 1990). In the ovary, Bone Morphogenic Protein (BMP) signals emanating from the niche are at a high level in GSCs thus preventing expression of Bam in the stem cells. However, in the CB, BMP signaling is low and Bam expression triggers development (Song et al. 2004). Each CB then undergoes 3 more rounds of mitosis with incomplete cytokinesis to form germline cysts. These cysts also contain a germline specific organelle called the fusome, which branches to every cell in a single cyst. The stage of a given cyst (2-cell, 4-cell, 8-cell or 16-cell) can be determined by how branched the fusome appears. Once this 16-cell cyst is formed it will start to bud from the germarium as an egg chamber and grow into progressively larger, more-developed, egg chambers. In the female germline, only one of the 16 cells will undergo meiosis and become a mature egg while the other 15 cells become polyploid nurse cells that grow and nurture the developing egg. As the egg chambers develop, the oocyte can be identified by staining for another RNA binding protein, oo18 RNA binding protein (Orb)(Christerson and McKearin 1994). During the final stages of egg chamber development, the nurse cells undergo cytoplasmic dumping (followed by apoptosis) to transfer their contents, which include RNAs, proteins, and organelles, into the oocyte (Buszczak and Cooley 2000). Within the mature oocyte there is cytoplasmic streaming

that can move material dumped by the nurse cells around to their proper location in the mature oocyte (Quinlan 2016)(Figure1.1A).

In the male germline, similar to the female germline, each GSC will undergo an asymmetric cell division resulting in one daughter cell remaining a GSC and the other cell becoming a gonialblast (GB) (Yamashita 2018). Each GB undergoes 3 more rounds of mitosis with incomplete cytokinesis to become a 16-cell cyst. Similar to the female germline, Bam is also a key differentiation factor in the male germline, however its expression turns on one cell division later in the 4-cell cyst (Gonczy, Matunis, and DiNardo 1997). Bam expression then continues in the 8-cell and 16-cell cysts until the onset of meiosis. Unlike the female germline, in males, all 16 spermatocytes of the 16-cell cyst will undergo meiosis resulting in the production of 64 mature spermatids per cyst. Following the completion of meiosis, sperm undergo a process called spermiogenesis resulting in drastic morphological change to produce the sperm head and tail while also removing cytoplasm to create essentially a nucleus attached to a tail primed to fertilize the egg it comes into contact with (Fig1.2). During this process the sperm remain together in a cyst and grow their tails in sync with each other. Once the tails are elongated the individualization complex extrudes the cytoplasm and breaks the cyst up into 64 individual sperm.

Studies by numerous labs (Beall et al. 2007; Kemphues et al. 1982; Mohammed et al. 2014)have identified many testis specific genes most of which are transcribed in the spermatocyte prior to the onset of meiosis. One unique feature of sperm development is that transcription shuts off at the onset of meiosis but the

process of spermiogenesis relies on a totally different set of proteins from those that function during the mitotic phases of development. A special set of testis-specific genes that are expressed in the spermatocytes are called meiotic arrest genes; these genes fall into two categories: the *always early (aly)* class or the *cannonball (can)* class (T.-Y. Lin et al. 1996). These classes of genes encode testis-specific TATA-binding-protein (TBP) associated factors (TAFs) (White-Cooper et al., 1998), and testis-specific meiotic arrest complexes (tMACs) (Beall et al. 2007), which activate the transcription of genes required for spermiogenesis. These spermiogenesis genes require extensive post-transcriptional gene regulation, which I highlight later in this chapter.

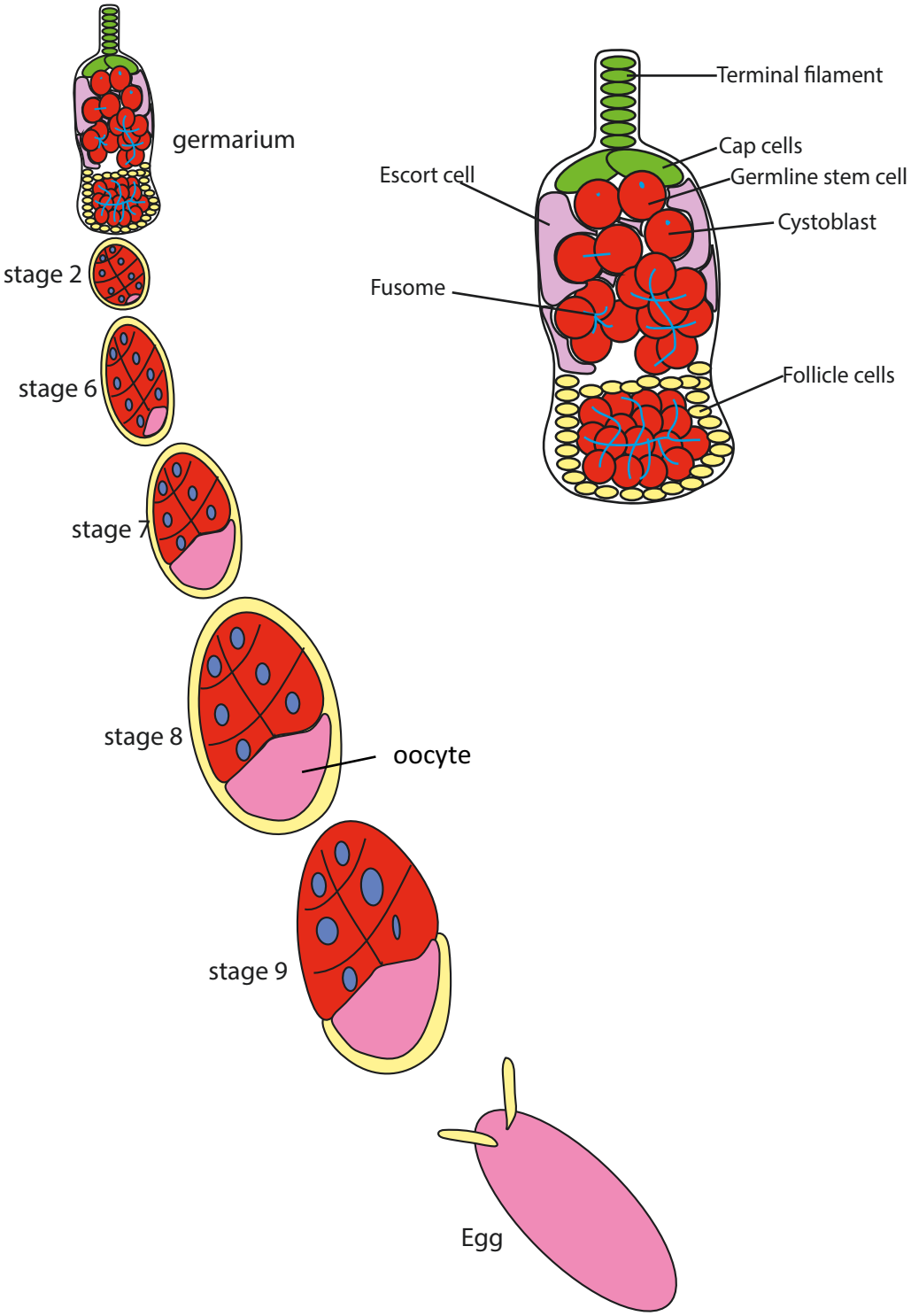

germarium
stage 2
stage 6
stage 7
stage 8
oocyte
stage 9
Egg
Terminal filament
Cap cells
Escort cell
Germline stem cell
Cystoblast
Fusome
Follicle cells

Figure 1.1: Drosophila female germline development

The germline stem cell niche is found at the apical tip of the germarium and consists of the terminal filaments and cap cells. Directly adhered to this niche are the GSCs. GSCs undergo an asymmetric cell division to produce a new GSC and daughter cystoblast. This cystoblast undergoes three rounds of mitosis with incomplete cytokinesis to form a 4-, 8-, and 16-cell cyst. These cysts are ensheathed by somatic escort cells until they are handed off to follicle cells at the onset of meiosis. The 16-cell cyst buds off from the germarium to produce an egg chamber. These egg chambers get progressively larger throughout development until they form a fully developed egg by stage 14. Each egg chamber consists of 15 polyploid nurse cells and one oocyte.

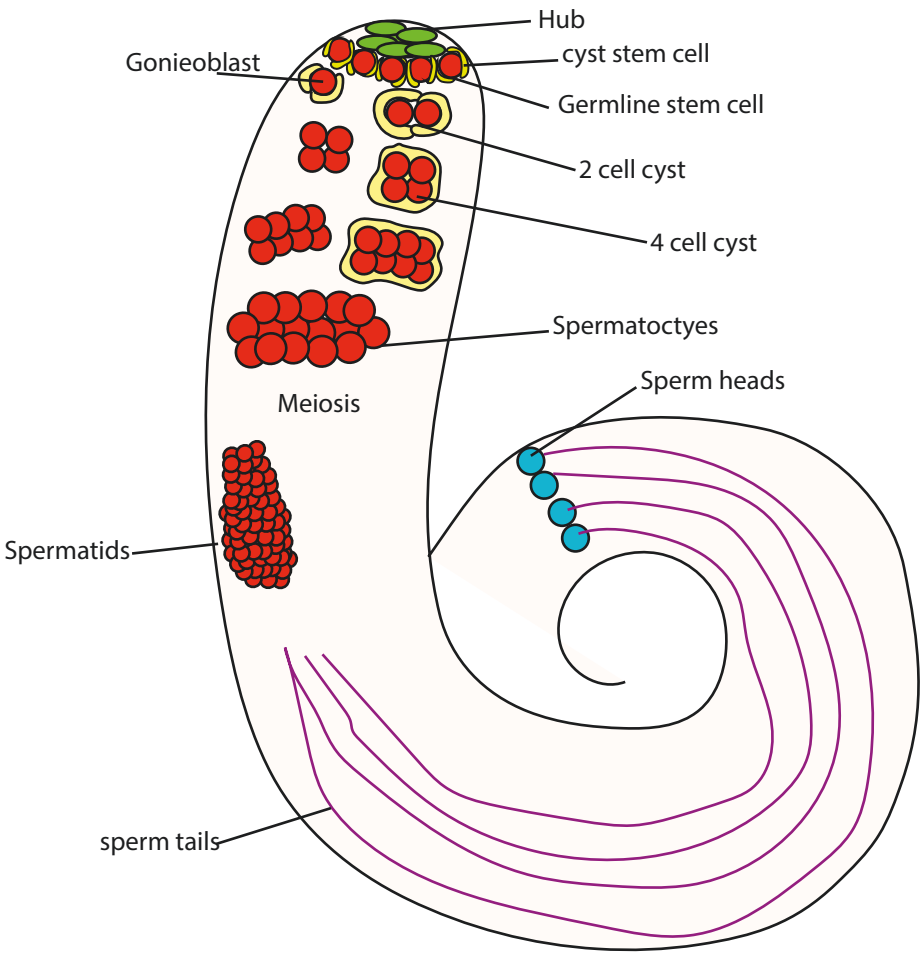

Hub
Gonieoblast
cyst stem cell
Germline stem cell
2 cell cyst
4 cell cyst
Spermatoctyes
Sperm heads
Meiosis
Spermatids
sperm tails

Figure 1.2: male germline development

The germline stem cell niche is found at the apical tip of the testis and consists of a cluster of post-mitotic hub cells. Directly adhered to this niche are the GSCs. GSCs undergo an asymmetric cell division to produce a new GSC and daughter gonialblast. This gonialblast undergoes three rounds of mitosis with incomplete cytokinesis to form a 4-, 8-, and 16-cell cyst. Each of these cysts are continuously ensheathed by 2 somatic cyst cells. 16-cell cysts undergo meiosis resulting in 64 spermatids per cyst. Following meiosis the 64 spermatids undergo spermiogenesis, which involves growing the sperm tails and extruding cytoplasm from the cysts. At the conclusion of spermiogenesis each spermatid becomes a sperm head with an attached sperm tail.

Significance of studying germline development

One question we are often asked is why study the *Drosophila* germline? As described above, there are many parallels between the development of the fly and mammalian germline. As will be described later in this chapter, not only do they both use the same chromosomal constitution to determine sexual identity, but their development relies on many of the same gene families and specialized mechanisms such as spermiogenesis and maternal gene products controlling early development since sperm provide no RNA to the zygote. Thus, our studies of the *Drosophila* germline will help shed light on the development of the mammalian germline in a more ethical manner than directly studying human gonad development in fetuses. When germline development goes wrong, the result is adult infertility, a condition that affects 1 in 8 couples in the United States and can be a very distressing condition to the couple affected by it (CDC 2020). Additionally, couples who struggle with infertility will often take on a huge financial burden to conceive a child. While we do have methods to help these families have children, very little is understood about human infertility at the genetic level aside from conditions that arise from larger chromosomal abnormalities such as Klinefelter syndrome and Turner syndrome, which account for only a small fraction of infertility cases ("How Many People Are Affected or at Risk?" n.d.; "How Many People Are Affected by or at Risk for Klinefelter Syndrome (KS)?" n.d.).

Tudor-domain-containing proteins of the germline

The tudor family of proteins is conserved across the animal kingdom for having essential roles in gametogenesis. The founding member of this gene family is the *Drosophila* Tudor protein, named after Henry VII, who struggled to have children and whose children had no offspring, since *tudor* mutants produce sterile offspring (Boswell and Mahowald 1985). Since the discovery of *Drosophila* tudor, many other tudor-domain-containing proteins have been discovered in flies and across the animal kingdom.

The grandchild-less phenotype noted in early *tudor* mutants was due to the absence of a germplasm. The germplasm is a special type of cytoplasm that specifies the germ cells in *Drosophila*, and similar types of specialized cytoplasm can be found in numerous species across the animal kingdom (Mukherjee and Mukherjee, n.d.). The germ plasm is both necessary and sufficient to produce germ cells. Transplantation experiments demonstrated that embryos with germplasm injected into various areas could develop an ectopic germline (Illmensee and Mahowald 1974). It was also demonstrated that injection of germplasm could rescue a germ-cell-less phenotype in embryos lacking a germ plasm. Within the *Drosophila* germ plasm, electron dense structures called polar granules are located next to mitochondria. In *tudor* mutant females, these polar granules are absent, suggesting that Tudor plays a role in polar-granule or germ-plasm formation (Boswell and Mahowald 1985).

Drosophila Tudor has 11 repeated tudor domains. Proteins belonging to the tudor family all have at least one of these domains and often have additional accessory domains (Handler et al. 2011). Many of the fly tudor domain-containing proteins have

direct mammalian orthologs. The mammalian homolog of tudor is Tdrd6 (Hosokawa et al. 2007).

The tudor domain consists of a core 60 amino acids whose function is to bind to dimethylated arginines and lysines through an aromatic cage in the domain (K. Liu et al. 2010). Additionally, many tudor-domain-containing proteins have what is known as an extended tudor domain, which contains an extra 180 amino acids (K. Liu et al. 2010). Interestingly, most germline tudor domains have these extended tudor domains. Another characteristic that separates some tudor-domain-containing proteins is the preference for binding to arginines vs lysines. Tudor proteins that function outside of the germline tend to act in the nucleus on histone regulation and to bind to lysine (Shanle et al. 2017). The extended tudor domain found in most germline expressed tudor-domain-containing proteins is thought to provide preferential binding to methyl-arginines due to the extra alpha helix and beta strands found in the extended domain (K. Liu et al. 2010).

Since the function of a tudor domain is to bind to other proteins, proteins that have tudor domains alone often act to scaffold protein complexes or recruit proteins with other functions. When a tudor-domain-containing protein possesses accessory domains (Table 1.1), it can both scaffold protein complexes and carry out additional functions. Many accessory domains allow tudor-domain-containing proteins to function in post-transcriptional regulation of RNA. Some of these domains, as noted in Figure 1.3, include RNA recognition motif (RRM), DEAD box, and Limkain, Oskar, and Tudor domain containing (LOTUS) domains. Others have accessory domains that allow them a second

mode of protein-protein interactions such as a zinc finger or Myeloid, Nervy, and DEAF-1 (MYND) domain (Handler et al. 2011).

A significant proportion of germline tudor domain-containing proteins function in the piRNA biogenesis pathway. (Anand and Kai 2012; Patil and Kai 2010; L. Liu et al. 2011) piRNAs are small RNAs whose function is to repress transposon expression. Some tudor domain proteins in the gonad such as Yb function in the somatic cells as part of the primary piRNA biogenesis pathway (Murota et al. 2014; Pandey et al. 2017). However, far more is known about how tudor-domain-containing proteins function in the germline piRNA pathway. In the germline there is a primary piRNA biogenesis pathway and a secondary piRNA biogenesis pathway also known as the ping-pong amplification cycle. Tudor-domain-containing proteins localize to the nuage (discussed below) where they play vital roles in both of these germline piRNA biogenesis pathways (Zhang et al. 2011; Patil et al. 2014).

In addition to functions related to piRNA biogenesis, some tudor-domain-containing proteins have been implicated in post transcriptional gene regulation based on their localization to the Processing Body, which is the granule where general mRNA decay and repression occurs (Sheth and Parker 2003). This role has been shown in flies with Partner of piwis (Papi) (L. Liu et al. 2011), and the chromatoid body (Chuma et al. 2003; Hosokawa et al. 2007; Vasileva et al. 2009)which is another area of general posttranscriptional regulation in mammals. In addition to general post-transcriptional regulation, specialized pathways such as nonsense mediated decay (NMD) also occur in these bodies and use tudor proteins such as TDRD6 (Fanourgakis et al. 2016, 6)

One of the more well studied RNA pathways using a tudor protein outside the piRNA pathway is snRNP biogenesis. This pathway requires the activity of Survival motor neuron (SMN) for proper function (Pellizzoni, Yong, and Dreyfuss 2002). SMN binds to dimethylated arginines on SM proteins that form a hexameric ring on a mature snRNP. SMN uses this binding to load SM proteins onto snRNAs to produce mature snRNPs that function in alternative splicing(Friesen et al. 2001). Moreover, numerous groups have shown that tudor-domain-containing proteins play a role in diverse RNA metabolism pathways in the germline and are necessary for fertility (Boswell and Mahowald 1985; Pek, Anand, and Kai 2012; Yabuta et al. 2011), thus making these proteins of great interest when studying germline development.

While tudor-domain-containing proteins seem to be present in endless RNA regulatory pathways in the germline, one common theme is that they are all involved in facilitating interactions among a larger RNP complex. These proteins can be found in virtually all RNA granules, which are membraneless organelles that will be described in depth later in this chapter. Lastly the conservation of many of these proteins across the animal kingdom (Handler et al. 2011) and their common role in ensuring proper fertility of an animal (Babakhanzadeh et al. 2020) make this protein family of particular interest for those studying the germline.

Fly Protein	Mouse ortholog	Accessory domains
Tudor	Tdrd6	
CG9925	Tdrd1	ZnF-MYND: DNA binding, protein protein interacting
CG9684	Tdrd1	ZnF-MYND: DNA binding, protein protein interacting
Vreteno		
Qin	Tdrd4	ZnF-RING: DNA/RNA binding, OB: nucleotide binding
Spindle-E	Tdrd9	DEAD: RNA helicase, Hel-C, OB, ZnF: nucleotide binding
Yb	Tdrd12	P-loop hydrolase: NTPase
Brother of Yb	Tdrd12	DEAD, P-loop hydrolase
Sister of Yb	Tdrd12	DEAD, P-loop helicase
Tejas	Tdrd5	LOTUS: Vasa and RNA binding
Tapas	Tdrd7	LOTUS, OB
Krimper	Tdrd1/Tdrd6	ZnF-CCCH: RNA binding
Papi	Tdrkh	KH: RNA binding, OB
Spoon	Akap1	KH, OB, A-kinase anchor
Tudor-SN	Snd1	SN-OB: RNA binding
CG15042	Tdrd1/Tdrd6	
Tdrd5l	Tdrd5	
Tdrd3	Tdrd3	RecQ: helicase
SMN	SMN1	
Splicing factor 30	Smndc1	

Table1.1 Tudor domain proteins in *Drosophila*

The first column lists all the known tudor-domain-containing proteins in *Drosophila.* In the second row is the corresponding mouse ortholog for each fly protein. Lastly the third row has accessory proteins for each fly protein as described on Flybase.

Post-transcriptional gene regulation

One level of gene regulation is simply whether or not an RNA is transcribed from its coding DNA sequence. However, regulation of that gene is far from over at the time of transcription. The term post-transcriptional gene regulation refers to regulation of a gene at any point after its transcription, usually at the level of regulating an RNA. It is this ability to regulate RNA after its transcription that allows cells to localize RNAs, trigger their degradation or translation, and fine tune many aspects of development.

Mechanisms of RNA regulation

mRNA molecules have structures and sequences that can be used to stabilize, localize, and regulate translation of the mRNA. During transcription all mRNAs (except histone mRNAs) get both a 5' cap and a 3' polyA tail. Both of these structures serve to protect the mRNA from degradation and to regulate translation. Translation initiation factors bind to the 5' cap to trigger translation, while removal of the cap targets that mRNA for degradation. The polyA tail serves to protect the 3' end of the mRNA but is also used to dynamically regulate translation. Shortening of the polyA tail can cause either degradation or silencing, while lengthening of the polyA tail is used to trigger efficient translation. It should also be noted that while the polyA tail can be shortened and lengthened, once the 5' cap is removed, it cannot be replaced(Moore 2005).

Just inside from the 5'cap and polyA tail are the 5' untranslated region (UTR) and 3'UTR, respectively. The sequences in both these regions are used to regulate mRNA

translation and stability. Most often factors that aid in translation initiation bind to the 5'UTR, while factors that regulate mRNA stability and localization bind to the 3'UTR. The 3'UTR can arguably be considered the most heavily regulated part of the mRNA. There are numerous proteins that bind to this region to repress or stabilize an mRNA. If an mRNA is not targeted for degradation but simply for translational repression, proteins such as cytoplasmic polyadenylation element binding proteins (CPEBs) can bind to the 3'UTR and trigger the addition of a polyA tail to de repress an mRNA(Hake and Richter 1994).

Aside from proteins, small RNAs also target the 3' UTR of mRNAs. All known types of small RNAs, siRNAs, miRNAs, and piRNAs, might bind to the 3'UTR in mRNAs as part of an RNA-induced silencing complex (RISC) to regulate their stability. All three populations of small RNAs can base pair with an mRNA. Once base paired, the RISC complex can trigger the immediate degradation of the mRNA or interact with other proteins to regulate the mRNA (Murota et al. 2014; Tang 2005). In the case of regulation, the GW182 protein can bind the Argonaut (Ago) protein and then recruit general repression complexes such as the decapping complex and the CCR4-NOT complex, which is responsible for deadenylation. Thus binding of a small RNA to the 3'UTR of an mRNA is just another mechanism that initiates the more general processes of post-transcriptional gene regulation, such as modulating polyA tail length or removing the 5' cap(Behm-Ansmant et al. 2006, 182; Fabian et al. 2011, 1).

In addition to these general mRNA decay and regulation pathways there are specialized pathways that can detect and degrade aberrant mRNAs. Many of these

pathways focus on the presence or absence of a stop codon. In nonsense mediated decay (NMD) a special set of proteins can detect a premature stop codon in an mRNA during translation based on the location of exon junction complexes, and funnel that mRNA into the normal decay pathways that take place in the processing body (described later)(Durand et al. 2007). Two other specialized decay pathways include "nonstop decay", for mRNAs missing a stop codon(Ge et al. 2010), and "no go" decay, for mRNAs with stalled ribosomes (Harigaya and Parker 2010). Here I have highlighted just a few of seemingly endless mRNA decay pathways, but the general mechanisms remain the same across most if not all pathways.

In addition to translational regulation, the 3'UTRS of mRNAs are often used for localization. The 3'UTR contains the sequences required for localization known as "zip codes. These zip codes allow RNA binding proteins to recognize the sequence or secondary structure of that region and attach the mRNA to motor proteins to transport them to the proper location in a cell. One cell type in which these sequences are particularly important is the central nervous system, where proteins such as beta actin need to be translated in distal parts of neurons; these regions can be up to an arm's (or a leg's) length away from the site of transcription in the nucleus. These sequences are also important in cells such as the oocyte, where RNA localization is heavily regulated to set up the body axes (Jambhekar and DeRisi 2007).

Importance of post transcriptional gene regulation in the germline

While post transcriptional gene regulation is crucial to germline development across the animal kingdom (Dallaire and Simard 2016), for the purpose of this section I

will focus on post-transcriptional gene regulation in the *Drosophila* germline. While development of the male and female germline exhibits some very dramatic differences and produces sex-specific gametes, both sexes rely heavily on post-transcriptional gene regulation in their germline development.

In the male germline, transcription is shut off prior to the onset of meiosis, but numerous proteins are needed after this shutdown for the specialized program of spermiogenesis (White-Cooper et al., 1998.). To get around this issue, the mitotic germ cells transcribe the mRNAs that are needed to translate these proteins later, but they repress their translation and store them until the completion of meiosis. Following the completion of meiosis, these special RNAs are de-repressed and allowed to be translated. Many of these genes have also been shown to be testis specific (Lin et al., 1996.)

In the female germline, RNA that is deposited into the oocyte is also heavily regulated at the post transcriptional level and will be described in detail below. In addition to the fact that these mRNAs have to be silenced during transport to the oocyte and often even after they make it to the oocyte, the localization of many of these mRNAs within the oocyte is also highly regulated (González-Reyes, Elliott, and St Johnston 1995). Since both localization and translational repression are aspects of post-transcriptional gene regulation, these pathways are critical for proper germline development and fertility of an animal.

RNA granules

RNA granules are membrane-less organelles that allow locally concentrated areas of proteins and RNAs. These granules often form through a process known as liquid-liquid phase separation. During this process proteins containing intrinsically disordered regions interact with each other to separate from the surrounding cytoplasm (Molliex et al. 2015; J. Smith et al. 2016). These proteins interact with other proteins and RNAs in a manner that allows all of these components to separate from the cytoplasm together in a granule. Additionally, these granules are often dependent on the presence of a seed RNA (Eulalio et al. 2007). There are numerous types of RNA granules found in virtually every cell in an organism and they can be found in the nucleus or the cytoplasm. Additionally, RNA granule proteins have been implicated in many diseases such as neurodegenerative disorders (Wolozin 2012). For the purposes of this introduction, we will focus on cytoplasmic RNA granules

While many types of cytoplasmic granules such as the processing body, U-body, and stress granule can be found in most tissues, there are also germline-specific RNA granules known collectively as germ granules such as the nuage (many animals), chromatoid body (mice), balbiani body (flies, fish), sponge body (flies), and the pING body (flies) (Table 1.1). These granules function in a wide array of pathways from small RNA biogenesis, RNA localization, maternal RNA deposition, storage, translational repression, and transposon repression, but all use proteins that accomplish these goals by repressing an mRNA. All of the above granules and their functions have been extensively reviewed (Voronina et al. 2011)

Granule	species	function
U body	All	snRNP assembly
P-body	All	mRNA repression
Nuage	All	piRNA production
Chromatoid body	Mammals	piRNA production and mRNA repression
Balbiani body	Flies, frogs	Mitochondrial inheritance
pING body	Male flies	Stellate repression
Sponge Body	Flies and worms	Similar to P-body

Table 1.1: RNA granules in the germline

The most widely conserved RNA granule in the germline is the nuage, which gets its name due to the cloud like shape it occupies around the nucleus. The nuage is best known for its role in piRNA biogenesis and transposon regulation(Lim and Kai 2007; Anand and Kai 2012; Chuma et al. 2003). It is in this granule that we find many tudor-domain-containing proteins. The nuage is home to RNA helicases such as Vasa as well as PIWI and Ago proteins, which are vital to the primary and secondary phase of piRNA biogenesis. Necessary for the primary phase of piRNA biogenesis are Piwi and Aubergine (Aub), while Argonaute3 (Ago3) functions in the secondary phase during ping-pong amplification. Nuage can associate with P- bodies, potentially to facilitate degradation of transposon RNAs. Localization of many nuage proteins relies on the symmetrical demethylation activity of Capsuleen (Csul)(Anne and Mechler 2005). Examples of proteins that need this methylation include Vasa, PIWI, and Ago3 (Kirino et al. 2009). Through these methyl marks, tudor-domain-containing proteins facilitate interactions and help ensure piRNA biogenesis.

The processing body or P-body is found in all tissues of an organism including the germline. As mentioned above, one possible function for the processing body is to receive transposon mRNAs from the nuage for degradation(Sheth and Parker 2003). The processing body is considered to be the general mRNA decay and repression granule in most cells and is often marked by proteins of the mRNA decapping complex such as Decapping protein 1 (Dcp1). For mRNAs that are targeted for degradation, they can be targeted directly to the P-body after recognition by general mRNA decay proteins, or

they can be targeted there from specialized decay pathways such as NMD(Durand et al. 2007). Additionally, if an mRNA is targeted to the P-body for temporary repression, the RNA is often deadenylated by the CCR4-NOT complex and can later have the tail lengthened by polyA polymerases such as Wispy to reactivate translation (Dufourt et al. 2017).

Similar to processing bodies are sponge bodies in the *Drosophila* egg chambers and C. elegans oocytes. These bodies harbor many of the same proteins that reside in P-bodies, and the two bodies were thought of interchangeably in early studies (Weil et al. 2012). Sponge bodies play a key function in repressing maternally deposited RNAs that need to remain translationally silent in the nurse cells (Wilsch-Bräuninger, Schwarz, and Nüsslein-Volhard 1997). Additionally, sponge bodies are not static structures and can move through nurse cells to assist in the localization of RNPs present in these bodies. Examples of RNAs that localize to the sponge bodies in flies are *grk* and *osk*, both of which are described in detail in the maternal RNA section. Live imaging shows that sponge bodies move through the ring canals from the nurse cells to the oocyte (Theurkauf and Hazelrigg 1998)(Snee and Macdonald 2009). Sponge bodies play a key role in anchoring mRNAs in the oocyte, such as bicoid (*bcd)* at the anterior and *gurken (grk)* at the dorsal anterior corner (Delanoue et al. 2007).

Lastly, a recently identified male-specific germ granule in *Drosophila* is the piNG body. This granule has been identified in spermatocytes and appears to be a special compartment of nuage. The piNG body contains many nuage components but appears as a larger granule next to the nuage. Additionally, factors occupying this granule can be

found in sub compartments often just the periphery of the body or the internal space of the body. For example, Vasa and Aub localize to the periphery while Ago3 localizes to the internal area of the granule (Kibanov et al. 2011). Functionally, these bodies are necessary for regression of Stellate retrotransposon product, which is known to occur via the piRNA pathway. Similar to the ovary nuage, proteins that localize to the piNG body require methylated arginines; this requirement suggests a similar mode of assembly regulated by tudor-domain-containing proteins as has been seen in the ovary (Kibanov et al. 2011).

The last important granule found in the germline is the balbiani body. This body is different from other germline granules that repress RNA, and it is the only granule to regulate cellular components other than RNAs. The balbiani body is a germline-specific granule that is found in the developing oocytes of animals such as flies and fish. The balbiani body is characterized by the presence of mitochondria, ER, and Golgi as well as RNAs. It is thought that the balbiani body acts as a filter to control mitochondrial quality and to ensure that only the best mitochondria are deposited into the germplasm to be inherited by the next generation(Cox and Spradling 2003). Initially the balbiani body sits at the anterior cortex of the developing oocyte, where four ring canals allow passage of RNAs, proteins, and organelles from the nurse cells to the oocyte. During development balbiani body material eventually breaks off from the anterior cortex and makes its way to the germplasm.

Maternal RNA Deposition

Maternal RNA deposition provides key oocyte mRNAs needed for embryonic development, and this process is conserved across the animal kingdom and is well documented in organisms such as frogs, and flies (Winata and Korzh 2018). In addition to deposition of RNAs, the mother also deposits proteins and organelles, the most important of which are the mitochondria. This process is essential since the zygotic genome doesn't produce RNA at the moment of fertilization; thus, products that the mother provides to the egg during maternal RNA deposition ensure early development and protein expression until the maternal-to-zygotic transition initiates zygotic transcription.

For the purpose of this introductory chapter, I will focus on maternal RNA deposition in the *Drosophila* germline, a process that has been extensively studied. Since the late 1970s and early 1980s, *Drosophila* has been a great model system for studying the regulation of maternally deposited RNAs and how those RNAs set up the embryonic axes (Johnston and Nüsslein-Volhard 1992). *Drosophila* embryos can be easily used to visualize the proper patterning of the anterior-posterior axis and dorsal-ventral axis. Additionally, work done by Nüsslein-Volhard and others determined numerous maternal-effect genes and genes required for this patterning (Berleth et al. 1988; Luschnig et al. 2004).

Axis specification in *Drosophila* relies on maternally deposited RNAs that are synthesized in the nurse cells and deposited into the oocyte. Once in the oocyte these RNAs are localized in a highly regulated fashion and locally translated. For example,

bicoid (bcd) is deposited into the oocyte and the mRNA localizes tightly to the anterior cortex of the developing oocyte. During early stages of embryogenesis, the *bcd* mRNA is translated and the protein diffuses to set up a gradient from high concentration of Bcd protein at the anterior to a low concentration of the protein as you move toward the posterior of the embryo (Driever and Nüsslein-Volhard 1988). This gradient activates gap genes such as *hunchback* to determine anterior fate of the embryo (Driever and Nüsslein-Volhard 1989). This proposed mechanism is supported by injection experiments done in *bcd* mutants where usually the anterior would not develop, but injection of the *bcd* mRNA rescues anterior development. Likewise, injection experiments also demonstrated that injection of *bcd* mRNA into other regions of the embryo could cause the anterior segments of the embryo to develop ectopically (Frohnhöfer and Nüsslein-Volhard 1986). Similarly, Nanos uses a system of decreasing protein concentration from poster to anterior to specify the posterior of the embryo. Thus, opposing gradients of Bcd and Nanos specify the anterior-posterior axis (Wang and Lehmann 1991).

Specification of the dorsal-ventral axis is largely due to the TGF-a homolog Gurken (Grk). During oogenesis, like other maternally deposited RNAs, *grk* is transcribed in the nurse cells and then deposited in the developing oocyte. During earlier stages in oogenesis, *grk* mRNA first gets localized to the posterior, where it is locally translated and facilitates specification of the posterior follicle cells (Gonzalez-Reyes and Johnston 1998). Subsequently, the oocyte nucleus moves to the dorsal anterior corner of the oocyte and so does the *grk* mRNA. Once at the dorsal anterior corner Grk protein is

again locally translated (F Shira Neuman-Silberberg and Sch, 1993.). Here the Grk protein activates the Egfr receptor on the surface of the dorsal anterior follicle cells to specify the dorsal end of the dorsal-ventral axis. During the last stages of egg development dorsal appendages, which are used for respiration, grow out of the dorsal side of the egg. In a *grk* mutant the eggs become ventralized, which can be seen as the dorsal appendages being absent or forming a single fused appendage. Mutants that affect Grk localization and cause it to spread more along the anterior cortex of the oocyte lead to the development of dorsalized eggs, which can be seen as eggs with ring-like dorsal appendages (F. S. Neuman-Silberberg and Schupbach 1994).

Prior to making it to their proper location, maternally deposited RNAs are heavily regulated post-transcriptionally. Once transcribed in the nurse cells they have to be translationally silenced until they make it to their final location. To accomplish this goal, maternally deposited RNAs are silenced while in the nurse cell cytoplasm(Wong and Schedl 2011; Nakamura et al. 2001). During transport along the cytoskeleton to the oocyte the RNAs remain silent and stabilized, until they are within the oocyte and translation is needed. While many of these RNAs go to completely different locations when they arrive in the oocyte, they share some common regulatory mechanisms (Kugler and Lasko 2009).

One way to prevent translation is to alter the binding of eukaryotic translation initiation factors (eIFs), which are responsible for cap-dependent translation. The most common way cells accomplish this inhibition is to prevent eIF4E from complexing with eIF4G and binding to the 5’ cap and initiating translation (Nakamura, Sato, and Hanyu-

Nakamura 2004). For example, the eIF4E binding protein Cup binds eiF4E and prevents it from initiating translation. Cup is necessary for both *osk* and *grk* repression(Nakamura, Sato, and Hanyu-Nakamura 2004; Clouse, Ferguson, and Schüpbach 2008). Interestingly, Cup is a general eIF4E binding protein; thus it has been suggested that its ability to regulate specific maternal RNAs works through other proteins that complex with these RNAs (Nakamura, Sato, and Hanyu-Nakamura 2004).

The *osk* RNA has Bruno response elements in its 3'UTR that allow the RNA-binding protein Bruno to associate with the RNA. Bruno can then interact with Cup to mediate *osk* repression(Nakamura, Sato, and Hanyu-Nakamura 2004). The *grk* RNA interacts with Bruno and also complexes with Squid, a heterologous RNP that recruits the Cup protein (Clouse, Ferguson, and Schüpbach 2008). These are just a few of many examples of repression of maternally deposited RNAs

Once in the oocyte and localized to the proper location, maternally deposited RNAs need to be de-repressed and translationally activated. The cytoplasmic polyadenylation-element binding protein Orb is one of these activators. Orb activates the translation of both *grk* and *oskar (osk)* RNAs in the oocyte (Castagnetti and Ephrussi 2003; Chang et al. 2001). I will use *grk* as the example for how this regulation works. Orb protein is present in the same locations where *grk* is localized. Orb binds to its binding sites in the 3'UTR of the *grk* mRNA. From there, Orb can recruit the protein Wispy, which is a cytoplasmic polyA polymerase, to lengthen the poly A tail of the *grk* mRNA and thus trigger its translation (Norvell et al. 2015).

While the above explanation focused on the regulation of *grk*, much more is known about *osk* and *nanos* than I highlighted in this introduction. While there is so much known about these RNAs and their functions in the oocyte, aspects of their regulation, and specifically what proteins are involved, remain to be studied especially in nurse cells.

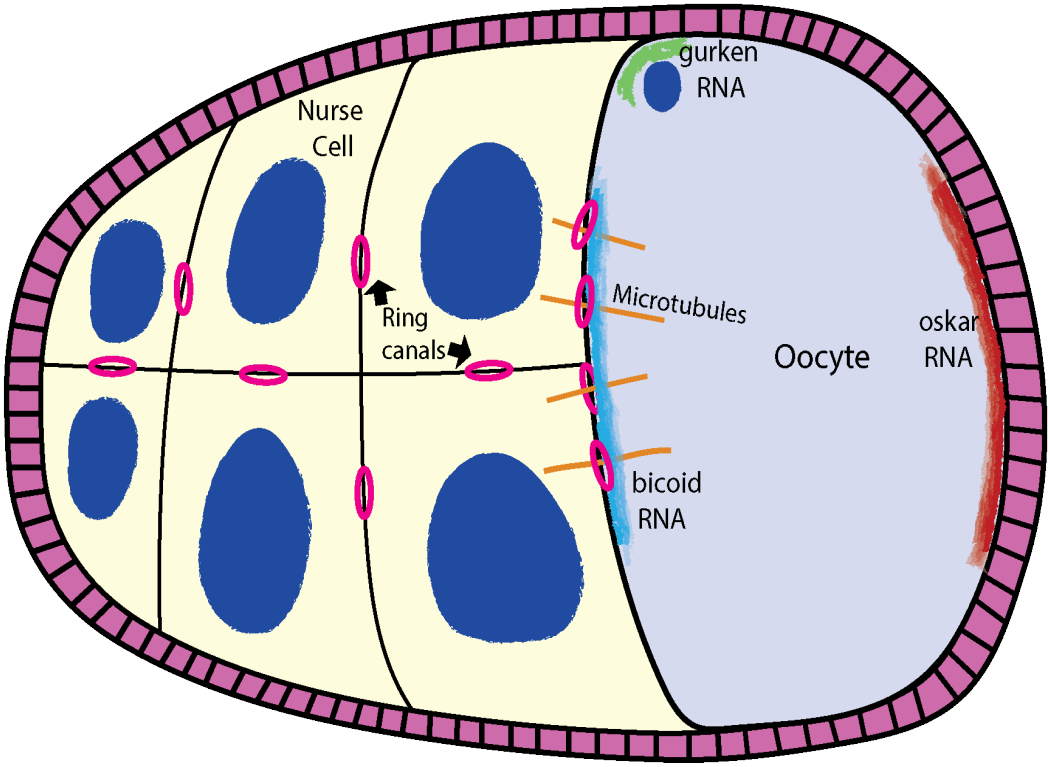

gurken
RNA
Nurse
Cell
Microtubules
Ring
canals
Oocyte
oskar
RNA
bicoid
RNA

Figure 1.3: Maternal RNA deposition

Developing egg chambers contain 15 nurse cells and an oocyte ensheathed by follicle cells. The 15 nurse cells are polyploid and function to produce RNAs, proteins, and organelles to deposit into the developing oocyte. RNAs in the nurse cells are packaged into RNPs and transported along the cytoskeleton through the ring canals into the oocyte. Once in the oocyte these RNAs then further localize to where they undergo localized translation. Examples include *bcd,* which localizes to the anterior cortex, *osk*, which localizes to the posterior cortex, and *grk,* which localizes to the dorsal-anterior corner of the oocyte.

Identification of *tdrd5l*

To identify targets of Sxl and sex-biased genes, a previous student in our lab, Shekerah Primus, conducted an RNAseq screen and found that the most differentially expressed gene in *Sxl* RNAi gonads was *CG15930,* now named *Tudor5-like (Tdrd5l).* Additionally, she found that *Tdrd5l* was highly expressed in male gonads compared to female gonads (Primus et al. 2019).

Since *Tdrd5l* is a Sxl target, Shekerah Primus tested whether Tdrd5l could promote male identity specifically in germ cells. When she expressed *Tdrd5l* in the germline of *tra* mutants, which have a masculinized soma but a female germline, she found that the atrophied-testis phenotype was rescued in 16% of flies and instead, these animals exhibited fully developed testes (Primus et al. 2019). This result suggests that Tdrd5l promotes male identity in the germline. It should be noted that these testes are not fertile since many sperm genes required for fertility are found on the Y chromosome and the flies in this experiment were XX flies.

Relation of Tdrd5l to its homologs

Tdrd5l gets its name from its most closely related homologs, TDRD5 in mammals and Tej in flies, which is thought to be the true TDRD5 homolog in flies. TDRD5 in mammals is essential for fertility in male mice (Yabuta et al. 2011). Similarly, Tej is also required for fertility in male flies, demonstrating the importance of TDRD5 proteins in the animal kingdom (Patil and Kai 2010). Both TDRD5 and Tej contain a tudor domain in their C-terminal half and a LOTUS domain in the N-terminal half. Both of their Tudor

domains are considered extended tudor domains, which is common of germline tudor proteins, and it is this domain that bares homology to Tdrd5l. While TDRD5 and Tej are predicted to have LOTUS domains, Tdrd5l is not predicted to have this domain. While there is no predicted domain in the N-terminal half of Tdrd5l, secondary-structure prediction shows there to be alpha helices in the N-terminal half; thus, it could possess an unknown function or a distantly related domain.

The mammalian TDRD5 represses retrotransposons in the male germline and post-transcriptionally regulates normal coding mRNAs in the male germline as well (Yabuta et al. 2011, 5). In flies, the only known function of Tej is to repress retrotransposons (Patil and Kai 2010). Our previous qRT-PCR experiments, however, which were designed to determine if transposon expression levels were changed in *Tdrd5l* mutants, showed that Tdrd5l does not share this function with Tej. Since Tdrd5l does not repress transposons, it is possible that the function of TDRD5 in mammals has been split between Tej and Tdrd5l in flies. If this hypothesis is in fact the case, it would not be the first time that a single tudor-domain-containing protein in mouse has been split into multiple fly homologs. The mammalian TDRD12 protein is split into 3 homologs in *Drosophila*: Yb, sister of Yb (SoYb), and brother of Yb (BoYb)(Handler et al. 2011). In the female gonad, Yb is expressed in the soma and is the marker of Yb bodies where piRNA biogenesis takes place in the soma. In the germline, both SoYb and BoYb function in the nuage where they also play roles in primary piRNA biogenesis (Hirakata et al. 2019). While all three of these homologs play similar roles, it does not rule out the

possibility that two homologs could play different roles in the germline as I predict is the case for Tej and Tdrd5l.

Chapter 2: Tdrd5l localizes to a novel germline RNA granule

Introduction

RNA granules are membrane-less structures that are used to locally concentrate RNAs and proteins for specific processes. They can be found in the nucleus or the cytoplasm and have been implicated in numerous cellular processes and diseases including development of the germline (Voronina et al. 2011). One functional aspect that many cytoplasmic RNA granules have in common is their role in post-transcriptional gene regulation, which is the regulation of RNA after it has been transcribed. Germline development depends heavily on post-transcriptional gene regulation, and this regulation relies on numerous types of RNA granules, some of which can be found in any tissue in an organism, such as the processing body, and others which are specific to the germline, such as the nuage.

The germline-specific granule in *Drosophila* and other organisms is known as the nuage, while in mammals, male germ cells also contain a granule called the chromatoid body (Vasileva et al. 2009). Both the nuage and chromatoid body are rich in tudor-domain-containing proteins. The nuage is the site of piRNA biogenesis, and piRNAs are vital to transposon repression (L. Liu et al. 2011). The germline is arguably the most important place to repress transposons to protect future generations. In mammals the nuage plays the same role in transposon regulation as in *Drosophila* while the chromatoid body is important mostly for regulation of coding mRNAs.

Finally, the most prevalent RNA granule that can be found in all tissue types across the animal kingdom is the processing body (P-body) (Tatosyan, Ustyantsev, and Kramerov 2020). The P-body is responsible for most general RNA regulation including

mRNA decay and translational repression. While all types of mRNA decay are permanent, translation repression is reversible, which allows P-bodies to function in diverse pathways. Often the polyA tails of an mRNA are removed, and this removal can target the mRNA to a P-body for storage until the polyA tail is re-synthesized, thereby allowing the mRNA to escape the P-body and undergo translation. Such a pathway is vital to the development of numerous tissues that rely on mRNAs being synthesized at a different time or place from where its translation or protein product is needed.

Similar to P-bodies are stress granules. When a cell undergoes stress, these granules accumulate mRNAs and grow in size in response to the stress. This sequestration allows the cell to halt most translation and conserve energy to survive a particular stress. This phenomenon can be brought on by chemical exposure, extreme temperatures, or starvation (Aguilera-Gomez et al. 2017; B. Kim, Cooke, and Rhee 2012; McEwen et al. 2005). In addition, stress granules contain many of the same proteins as P-bodies but also contain translation initiation factors (Balagopal and Parker 2009).

In addition to the major granules mentioned above, there are some other germline-specific granules, some of which are found in multiple species and some of which are species-specific. These include the P-granule in *C. elegans* and the balbiani body in many animals including *Drosophila,* both of which play important roles in germline development. Since previous data have shown that Tdrd5l localized to an RNA granule, understanding what type of granule could help shed light on the function of Tdrd5l.

Methods

CRISPR tagging

CRISPR Cas9 tagging was done using reagents from the Fly CRISPR group (Gratz et al. 2015). Guide RNAs were created to recognize regions near PAM sites for each specific construct. The gRNA sequences are as follows, N terminal GFP tag: ggtgtggtggatttcgcggatgg internal FLAG and HA tags: GACGGATGGTTACATTGTCA and C terminal FLAG tag GATTTCGCATTCGTTCCATAG. For internal and N-terminal tags, one gRNA was injected along with the repair construct. For the C-terminal tag, two gRNAs were injected. All gRNAs were inserted into the pU6 chi-gRNA plasmid for injection (Gratz et al. 2015).

Repair templates were constructed by using Gibson assembly to assemble the 5' homology arm, tag, removable DsRED cassette, and 3' homology arm. Each homology arm contains 900-1000 bps of genomic sequence immediately upstream (5') and downstream (3') of the tag. The assembled fragment was TA cloned into the pCR2.1 plasmid, and the PAM site was mutated using Round the horn PCR. gRNA plasmids and repair constructs were injected into *nanos-Cas9* flies.

Following injection done by Best gene, injected embryos were shipped back to lab and are called G0 flies. Individual G0 flies were mated to Fm7c flies and their progeny were screened for expression of DsRED, which indicated integration of the tag and DsRED cassette. DsRED-positive flies were then crossed to flies containing the piggyBac transposase to remove the DsRED cassette and bring the tag in frame with Tdrd5l. Male progeny from the piggyBac cross were then mated with FM7c females.

DsRED-negative female progeny from that cross were then used as founders for the final tagged-allele stock.

Sources and genotypes of flies

All fly stocks were obtained from the Bloomington stock center or from the noted sources. *UAS-SMN* was obtained from Joe Gall (J.-L. Liu and Gall 2007), *Dcp1:YFP* obtained from (M.-D. Lin et al. 2006), HA: Tdrd5l Bac (Primus et al. 2019). All UAS-RNAi was driven using *nos*-GAL4 stock.

Immunofluorescence

Dissected gonads were fixed in 4% formaldehyde in PBS + 0.1% TritonX (PBTx) for 20min at room temperature while rotating. Following fixation, the tissue was rinsed 2x in PBTx and washed 2x for 10min in PBTx. Washed tissue was blocked in PBTx + 0.5% BSA (BBTx) plus normal goat serum (NGS) for 30min. Tissue was incubated in primary antibodies overnight while rocking at 4oC in BBTX plus NGS. Antibodies used were rabbit anti-Vasa (1:10,000, Ruth Lehman), guinea pig anti-Traffic Jam (1: 1000), rat anti-HA (1:100, Roche), rabbit anti-Me31b (1:5000, A. Nakumara), mouse anti-FLAG (1:500, Sigma), guinea pig anti-Tdrd5l (1:2000-1:5000). Samples were rinsed 2x in PBTx and washed 2x for 10min in PBTx. Samples were then stained in secondary antibody, goat IgG (Invitrogen) overnight at 4oC while rocking. Samples were then incubated in DAPI for 10min while rocking at room temperature and mounted in DABCO mounting media. Confocal microscopy was conducted using a Zeiss LSM 700 or LSM 800 with airyscan. Images were processed using Zen and Fiji software.

Tdrd5l antibody production

A peptide antibody was raised in Guinea pigs against amino acids 69-88 of Tdrd5l (sequence) which is highly specific to Tdrd5l. The peptide was synthesized by BioMakit and conjugated to KLH carrier protein. Conjugated peptide was injected into 2 guinea pigs and serum collected by Pocono Rabbit farms. Half of the final bleed serum was peptide purified using the Thermo Fisher sulfolink immobilization kit for peptides.

RNaseA assay

Wildtype testes were dissected and incubated in PBTx for 20min to permeabilize the tissue. Testes were washed 3 times in Sneider's media to remove detergent. Control testes were incubated in Sneider's media plus PBS for 30min and RNase testes were incubated in Sneider's media + 1% RNaseA. After RNase or PBS treatment, testes were fixed and stained as described above.

Results

Tagging of Tdrd5l

To get a better understanding of the true regulation and expression pattern of Tdrd5l, I created a GFP-tagged allele at the endogenous locus using CRISPR/Cas9. To add this tag, I specifically used the CRISPR reagents from the fly CRISPR consortium (Gratz et al. 2015). Following removal of the dsRED reporter, the tag is brought into frame with your protein of interest. Since the only predicted domain in Tdrd5l is in the C-terminal half of the protein, we chose to tag the N-terminus to avoid potential disruption to

protein folding (Figure2.1). Imaging of GFP-Tdrd5l testes showed no GFP expression with endogenous fluorescence or antibody staining, as seen by total absence of green fluorescence within the dotted outline of the testis (figure 2.2A, n=20 testes). Interestingly all females heterozygous for the GFP-Tdrd5l tag displayed GFP-positive granules in the germline, while homozygous females were devoid of GFP staining similar to males as shown by absence of green fluorescence in the outlined ovariole. (Figure 2.2 B-C; N= 25 per genotype).

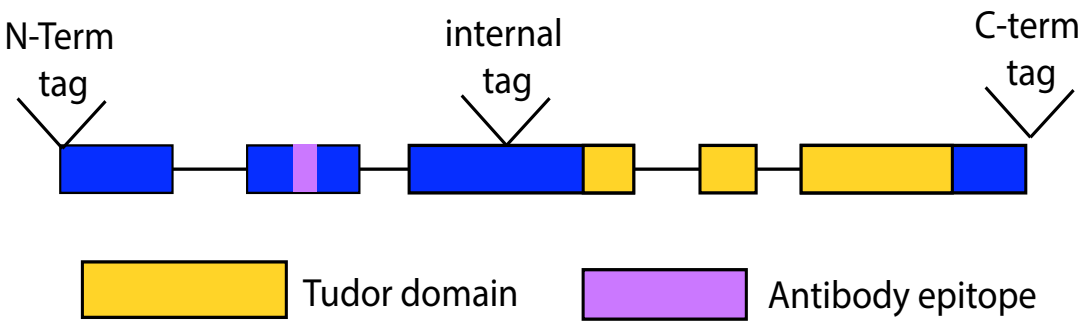


Figure 2.1 Schematic Diagram of *Tdrd5l* Locus

The only predicted domain in Tdrd5l is a tudor domain depicted in yellow. Depicted in purple is the location of the epitope used to create the Tdrd5l antibody. Locations of the tags are also noted as N-terminal, internal, and C-terminal.

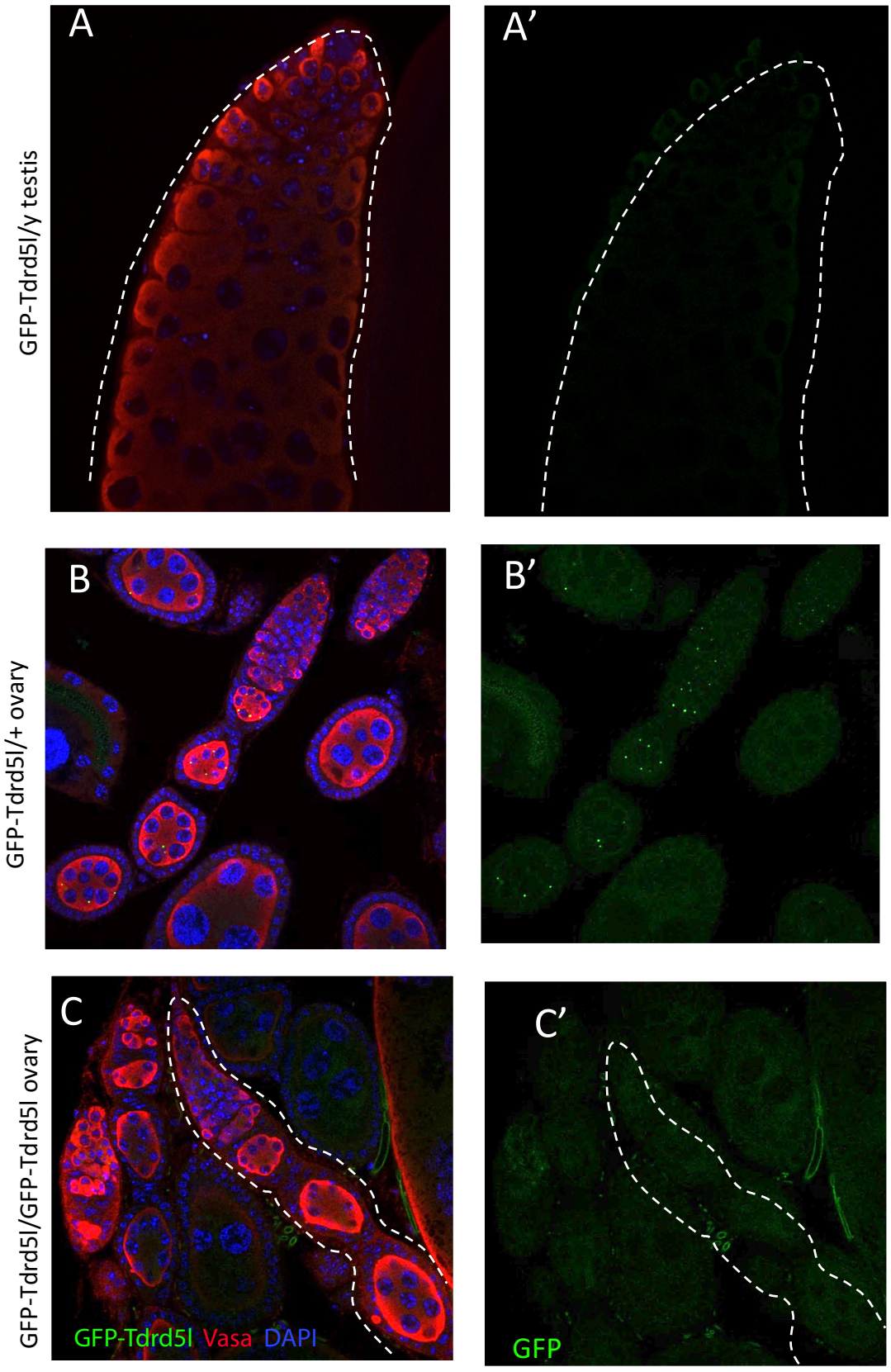

A
A'
GFP-Tdrd5l/y testis
B
B'
GFP-Tdrd5l/+ ovary
C
C'
GFP-Tdrd5l/GFP-Tdrd5l ovary
GFP-Tdrd5l Vasa DAPI
GFP

Figure 2.2 N-terminally tagged Tdrd5l does not localize to granules without a wildtype copy

(A) *GFP-Tdrd5l/Y* testes show no GFP expression or noticeable Tdrd5l granules. (B) *GFP-Tdrd5l/+* ovaries express the GFP-tagged construct, which is seen localized to discrete granules while in (C) *GFP-Tdrd5l/GFP-Tdrd5l* females there is no GFP expression, similar to what is observed in the testis (A).

These data suggested that the N-terminal tagged Tdrd5l protein was unable to localize to the granule in the absence of wild-type protein. To test this hypothesis, we analyzed *GFP-Tdrd5l* expression when present in trans to a *Tdrd5l* mutant allele in females. Similar to when *GFP-Tdrd5l* was homozygous in females, when expressed over a mutant allele of *Tdrd5l* there was still no GFP staining detected in the outlined ovary. These results suggested that GFP-Tdrd5l requires a wild-type copy of the protein to localize to granules (Figure 2.3).

Next we asked whether a wild-type copy of Tdrd5l could rescue GFP-Tdrd5l localization to granules in males. To test this idea, we used males carrying a duplication of the *Tdrd5l* region of the X chromosome inserted onto the 3rd chromosome. We examined testes from males bearing *GFP-Tdrd5l* on the X chromosome and the *Tdrd5l*-containing duplication on the 3rd chromosome. Imaging of these gonads revealed a partial rescue of GFP-Tdrd5l localization to granules. In these gonads we observed smaller granules in the early germline as we expected but none of the larger granules that appear hollow for Tdrd5l staining in spermatocytes that we had identified using a previously made HA-tagged BAC construct (Primus et al. 2019) (Figure 2.4).

Lastly, we asked if the GFP-Tdrd5l construct behaves like a *Tdrd5l* mutant allele. To test this possibility, since the null allele by itself has a weak fecundity phenotype, we took advantage of the genetic interaction between *Tdrd5l* and the mRNA deadenylase encoded by *twin* (discussed in chapter 3). *twin* RNAi driven by *nos*>GAL4 in a wildtype background displayed no morphological or fertility defects in males or females. However, when *twin* was knocked down in the germline of *Tdrd5l* null-mutant flies, both

males and females were sterile, and females displayed germline loss and germ-cell tumors. We found that the N-terminal *GFP-Tdrd5l* allele behaves like a *tdrd5l* null-mutant allele in the *twin* genetic interaction assay. From this result we can conclude that adding a tag to the N-terminus of Tdrd5l creates a mutant allele, and that normal function might depend on Tdrd5l localizing to its granule (Figure 2.5).

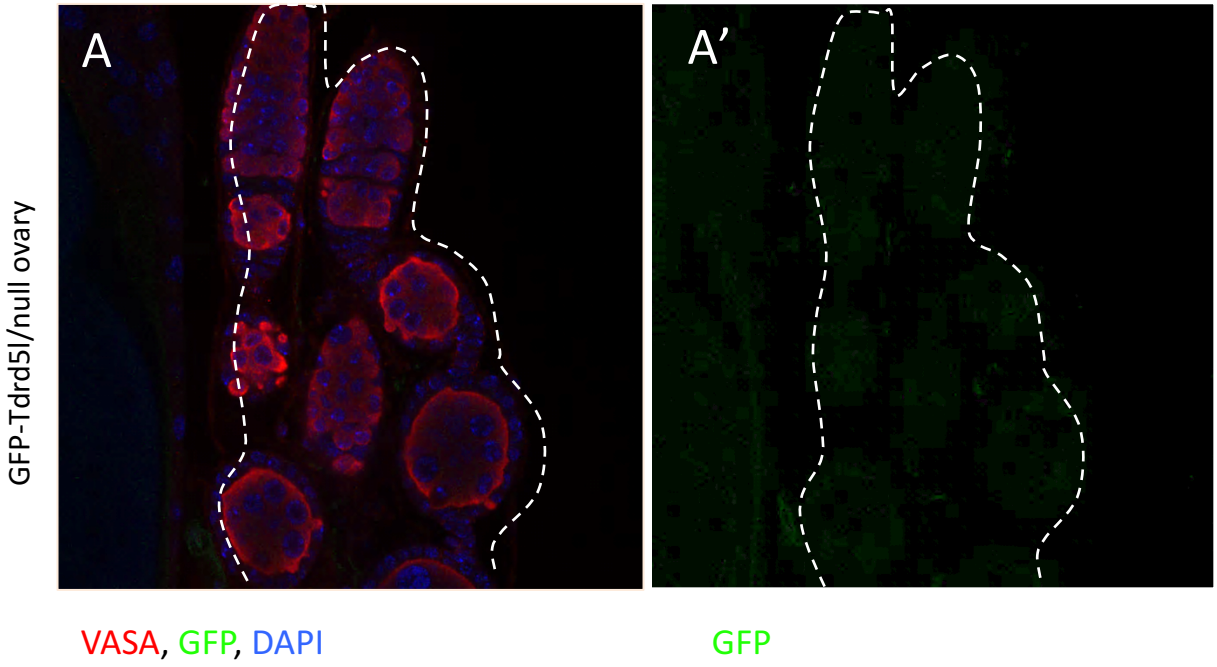


Figure 2.3 GFP-Tdrd5l does not localize to granules in the presence of a null allele

(A) No GFP expression is detected in ovaries from flies that are heterozygous for *GFP-Tdrd5l/null*

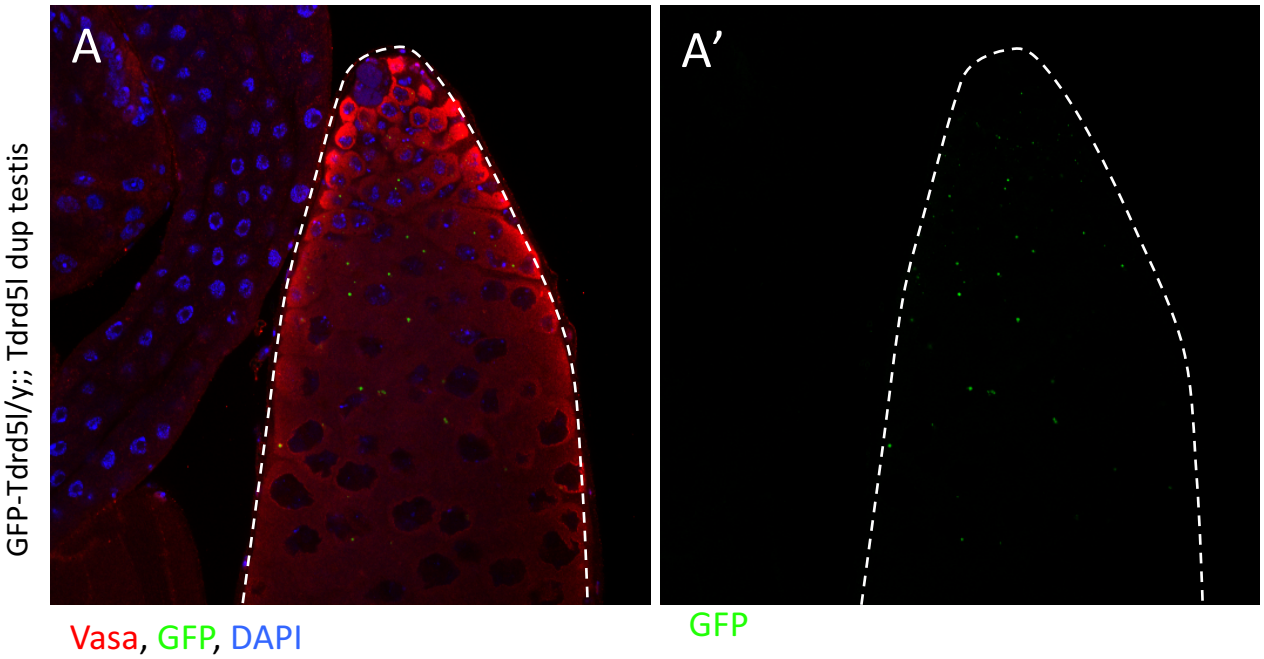


Figure 2.4 Wildtype Tdrd5l rescues localization of GFP-Tdrd5l

(A) GFP expression is seen in granules in testes from flies that express a wildtype duplication of *Tdrd5l* on the 3rd chromosome in addition to the *GFP-Tdrd5l* allele on the X.

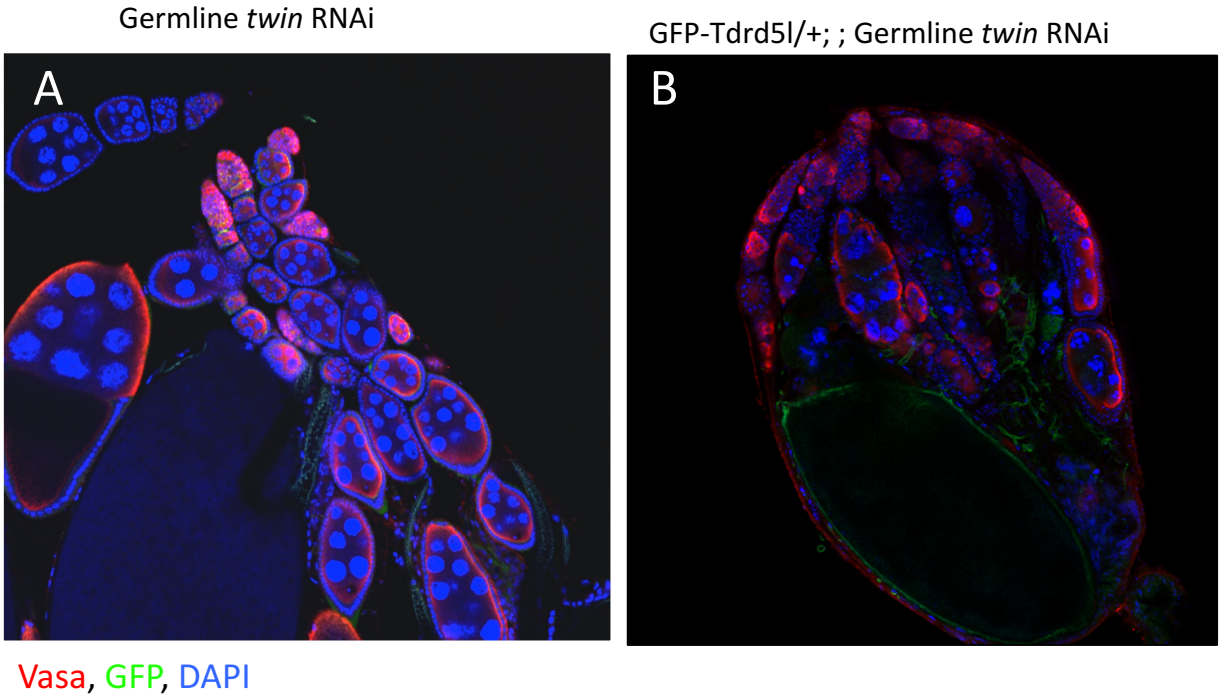


Figure 2.5: GFP-Tdrd5l behaves as a mutant allele

(A) Little to no phenotype is present in ovaries from wildtype flies and *twin* RNAi in the germline, however when *twin* is knocked down in the germline of *GFP-Tdrd5l* flies (B) germline tumors form similar to when *twin* is knocked down in a *tdrd5l* mutant fly (chapter 3).

To allow us to visualize Tdrd5l in a wildtype context, we took two other approaches. First, we created an antibody against Tdrd5l and second, we created additional epitope-tagged alleles of *Tdrd5l*, this time tagging an internal site and the C-terminus. The antibody we created was directed against a specific peptide that is unique to Tdrd5l. Validation of the antibody showed that staining worked well in males and identified all the granule species we had previously seen. These granules included the smaller early germline granules and the later large granules that are devoid of Tdrd5l staining in the center (Figure 2.6A). In females I identified granules in ~10% of ovaries which was likely due to the low expression level of Tdrd5l in the female germline (Figure 2.6B). Additionally, we were able to detect Tdrd5l in testes by western blot (Figure 2.6C).

Since tagging the N-terminus of the protein resulted in a mutant allele of *Tdrd5l*, we next created a C-terminal endogenous FLAG-tagged allele using CRISPR/Cas9. While we were able to observe some FLAG staining in the male germline, some testes had no granules and others had ~10% of the total number of granules per testis seen with the antibody (Figure 2.7; N= 13). Additionally, we rarely observed the larger granules that appear hollow for Tdrd5l staining which I usually observe in the spermatocytes. Thus, the C-terminal tagged allele also did not recapitulate normal expression and was therefore similar to what was seen with the N-terminal-tagged alleles.

To create an internal tag, we utilized a secondary structure prediction (Drozdetskiy et al. 2015)to identify a location in the protein where a tag was least likely to disrupt folding. Even though there are no predicted domains in the N-terminal half of the protein, there are predicted alpha helices as well as short stretches of disorder that might be important for localization to a granule. Due to these features, we chose to add the internal HA tag just following the last disordered stretch but before the predicted tudor domain. Anti-FLAG staining of both testes and ovaries shows that FLAG-tagged Tdrd5l localizes to germline granules (Figure 2.8). In the male germline FLAG-Tdrd5l localizes to granules of the same size and distribution as seen in Anti-Tdrd5l staining (Figure 2.8 n= 24). In the male germline we still observe more small granules in the early germline and an average of 1 large granule per cell in the later mitotic germ cells. In females we observed FLAG-Tdrd5l expression in the germarium where it localized to granules. I also observe some granules in the developing nurse cells as well. Additionally, there was some diffuse non-granule staining seen in the female germline (Figure 2.8B).

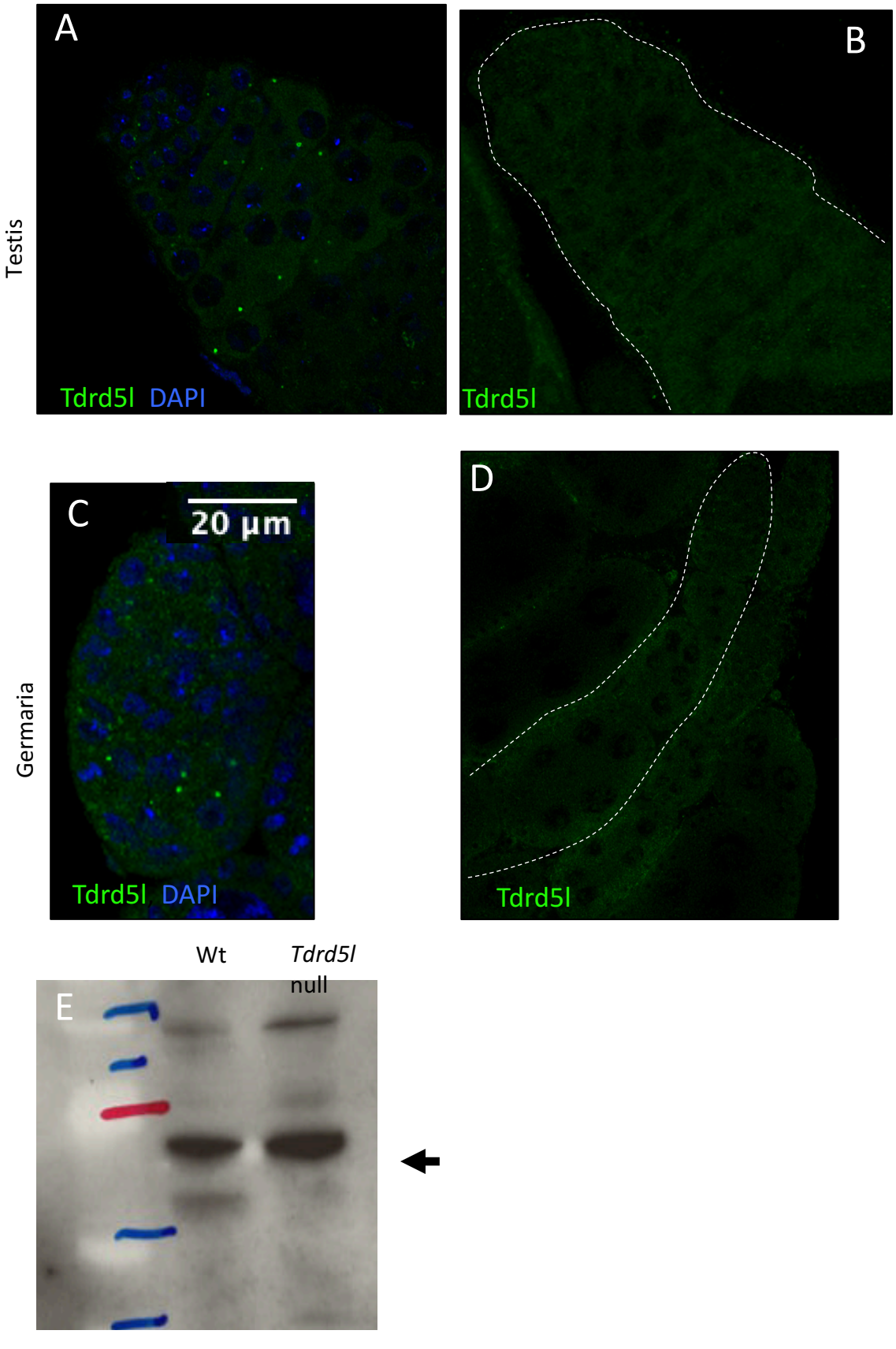

A
B
Testis
Tdrd5l DAPI
Tdrd5l
C
20 µm
D
Germaria
Tdrd5l DAPI
Tdrd5l
Wt
Tdrd5l null
E

Figure 2.6 Tdrd5l antibody specifically recognizes Tdrd5l in the germline

(A) The Tdrd5l antibody recognizes granules in the male germline and in the (C) female germline. No staining is seen in *Tdrd5l* mutant males (B) or (D) *Tdrd5l* mutant females (E) Western blot analysis shows the antibody recognizes a band specific to Tdrd5l in wildtype flies, that is absent from *tdrd5l* nulls as marked by the black arrow. What are the sizes of the markers?

Flag tagged Tdrd5l testis

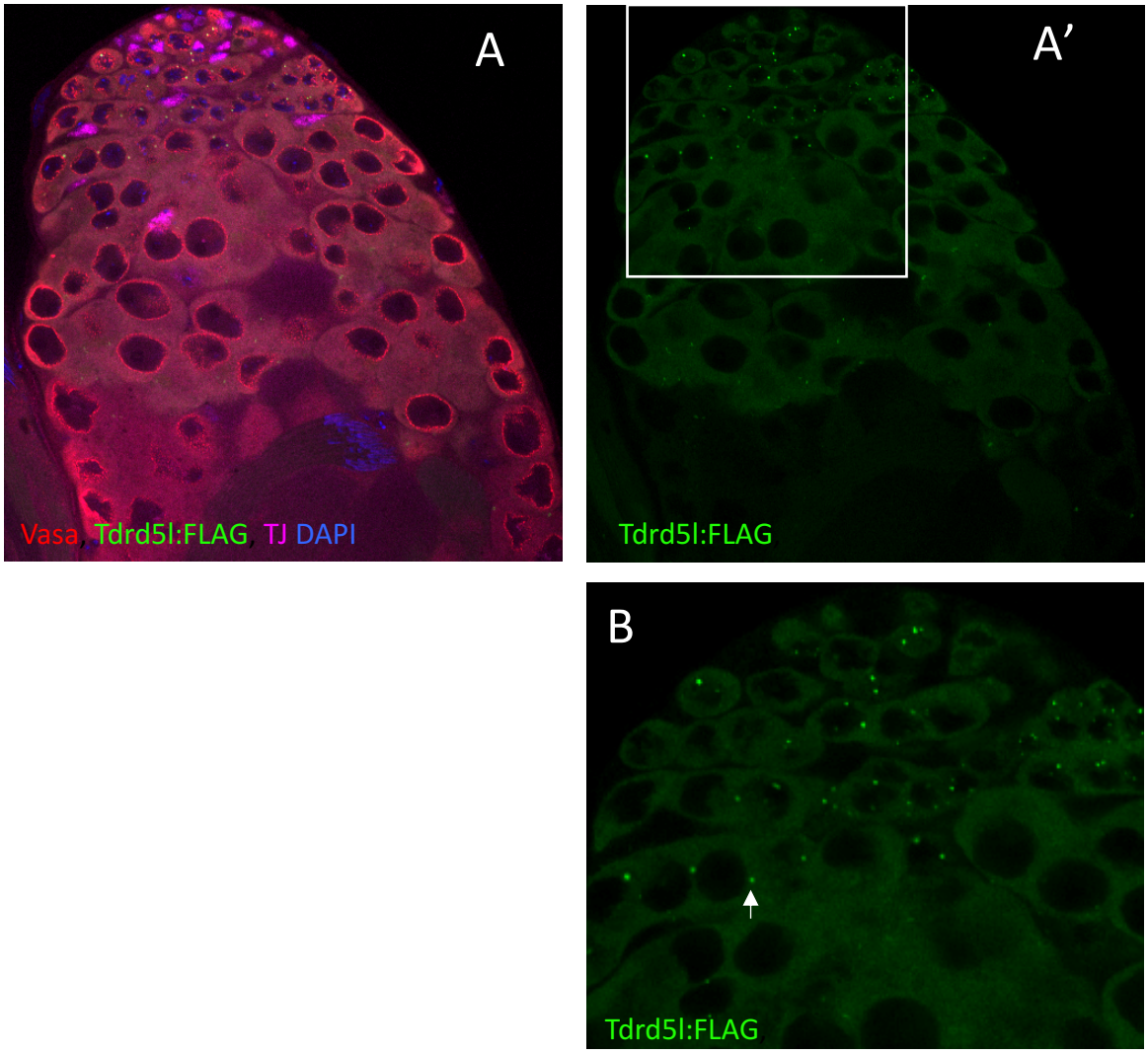


Figure 2.7: C-terminal tagged Tdrd5l does not localize properly

C-terminally tagged Tdrd5l does not recapitulate Tdrd5l antibody staining as seen in (A) Blow up of the boxed region in A' is shown in (B). In (B) the granules are much smaller than seen with antibody staining as marked by the arrow, and there are fewer granules overall.

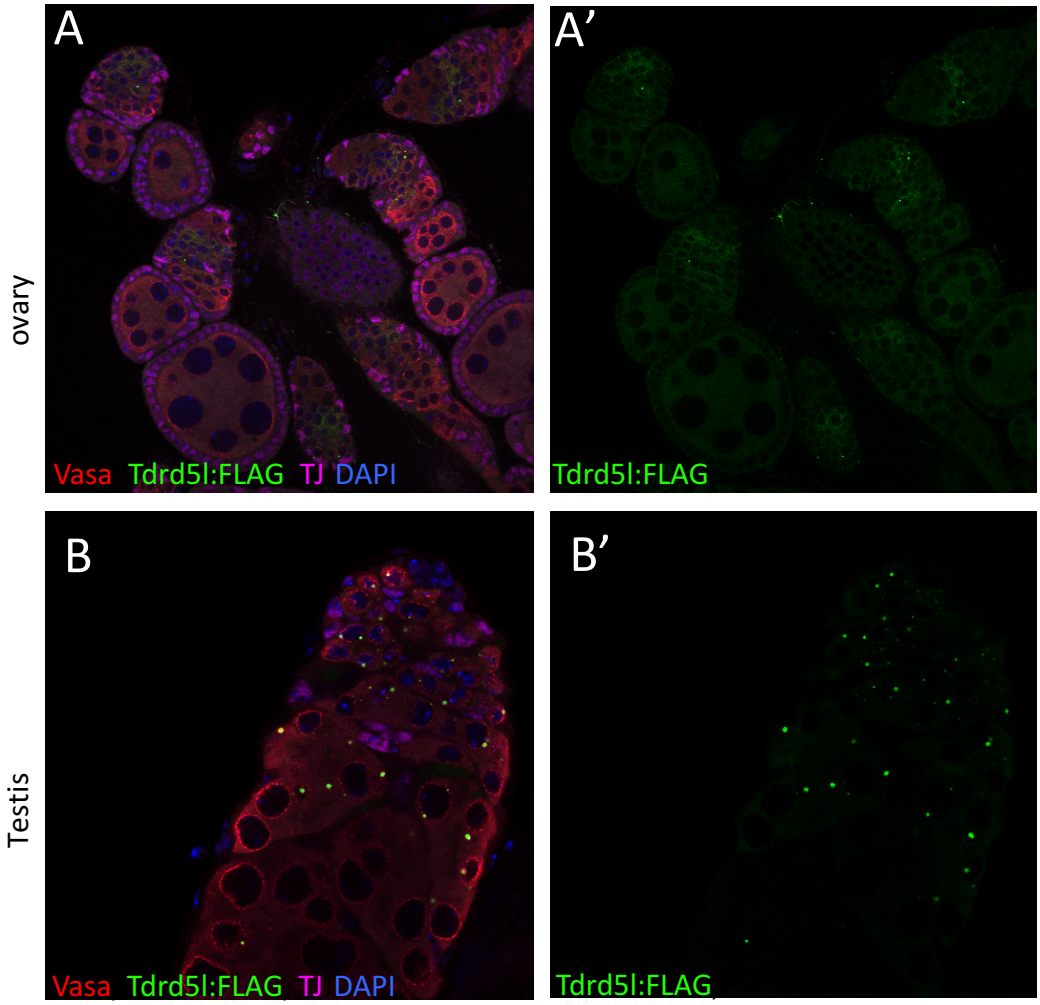


Figure 2.8: Internal Flag tagged Tdrd5l localizes to germline granules

Internal Flag tagged Tdrd5l recapitulates antibody staining in males (A) and in females (B)

Tdrd5l expression pattern in the germline of testes and ovaries

Tdrd5l is most highly expressed in the premeiotic germline in the male gonad. Tdrd5l protein expression in the male germline is first seen in the testis germline stem cells (GSCs). In the GSCs we observed a mixed population of both small (100nm) and large (900nm- 1micron) Tdrd5l containing granules as well as some faint diffuse cytoplasmic staining. As the germline develops from GSCs to spermatocytes, the granules become predominantly larger granules in the spermatocytes and the smaller granules become a smaller fraction of the granules present per cell (Figure 2.9A, N= 23 testes). With entry to meiosis Tdrd5l expression turns off.

In the female germline, Tdrd5l is first expressed in the germarium and notably absent from the GSCs, but it is expressed one cell division later in the cystoblasts (CBs) (Figure 2.9B). This difference in GSC expression is consistent with the fact that SXL Is highly expressed in female GSCs and likely repressing Tdrd5l in those cells. Tdrd5l continues to be expressed in the first cyst to bud off of the germarium and is expressed in the egg chamber nurse cells until stage 6 (Figure 2.9B). Contrary to the males, there is no noticeable change in Tdrd5l granule size with development; additionally, there is diffuse cytoplasmic expression of Tdrd5l in the germarium (Figure 2.9B).

Since tudor-domain-containing proteins often are involved in RNA regulation, we tested whether RNA was required for Tdrd5l granule localization. To remove RNA, we treated gonads with RNase A while incubating in Schneider's media. Following fixation, gonads were stained for Tdrd5l and compared to gonads treated with PBS instead of RNaseA. Testes treated with RNase A had a dramatic decrease in Tdrd5l

granules compared to controls. I observed a 95% decrease in smaller granules and a 90% decrease in the larger granules that are hollow for Tdrd5l staining (Figure 2.10 N= 10 per genotype)

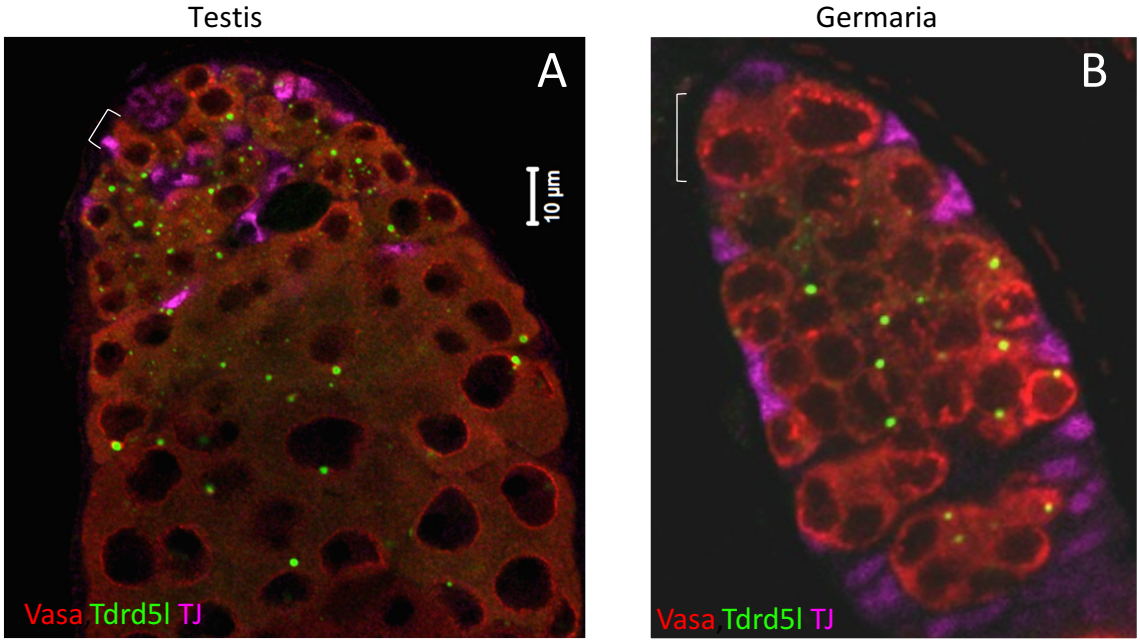


Figure 2.9 Expression pattern of Tdrd5l in males vs females

(A) In the male germline Tdrd5l is expressed in the germline stem cells marked by brackets and then continues until the onset of meiosis. (B) in the female germline Tdrd5l granules are absent from the germline stem cells marked by brackets. Tdrd5l expression begins in the cystoblasts and continues in the rest of the germarium.

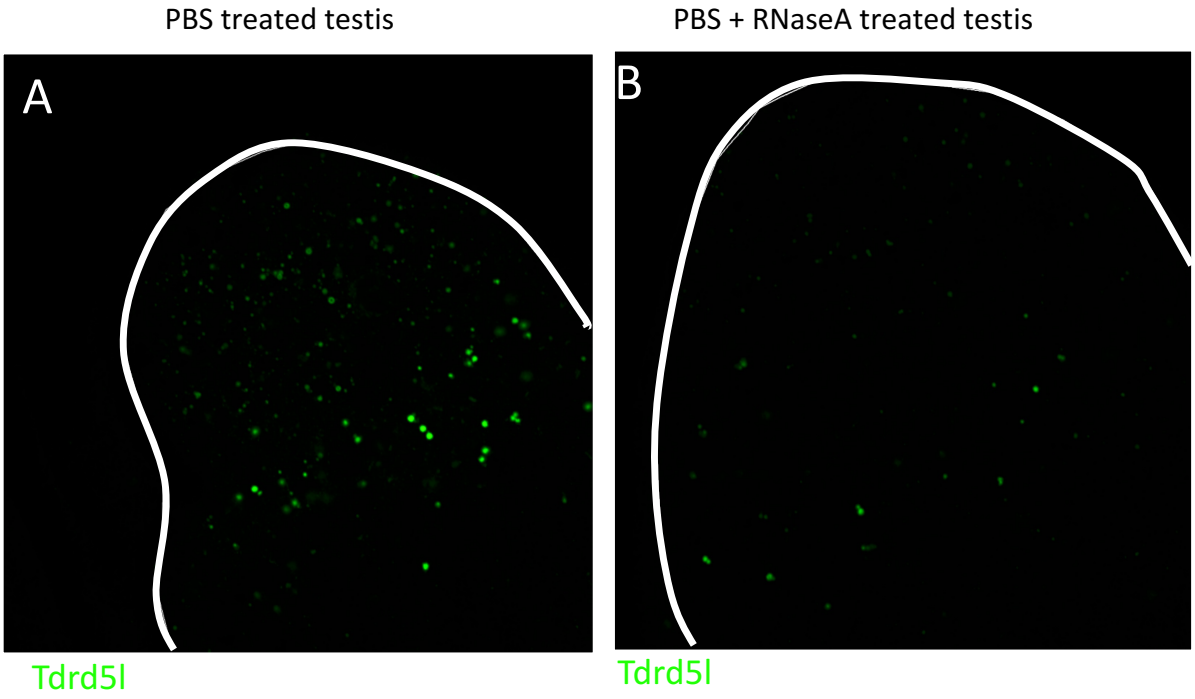
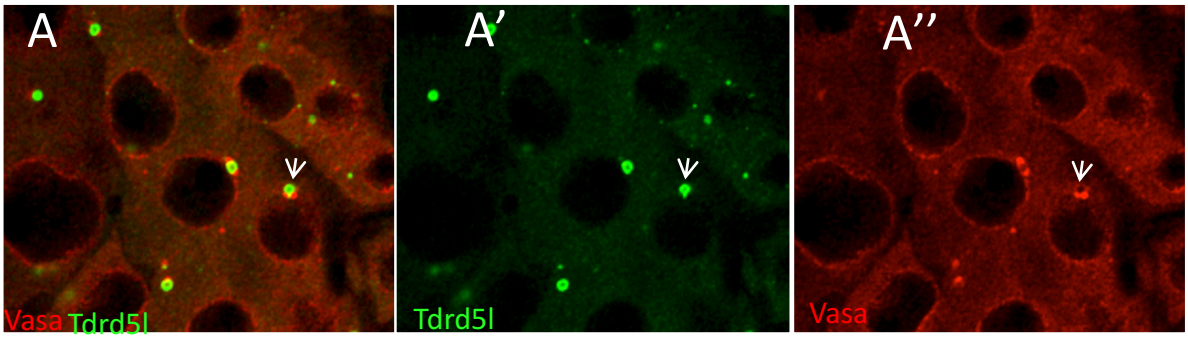


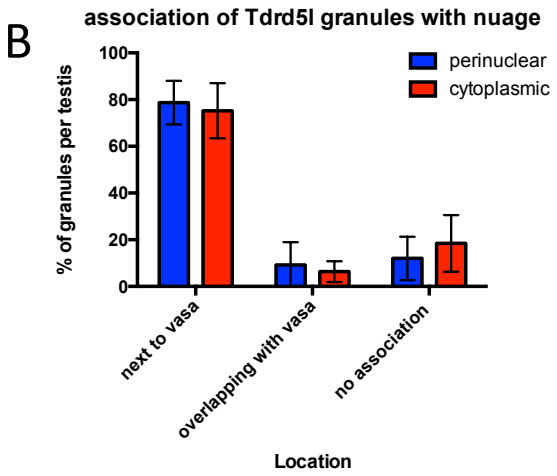
Figure 2.10: RNA is required for Tdrd5l granule Assembly

In an RNase A assay, (A) testes treated with PBS along displayed wildtype levels of Tdrd5l granules in the germline. (B) In testes treated with PBS plus RNase A there were diminished amounts of Tdrd5l granules in the testis. In both A and B the testes are outlined by a white line.

Tdrd5l localizes to a novel germline RNA granule

To determine what type of granule Tdrd5l occupies, we co-stained for Tdrd5l and proteins known to localize to different types of RNA granules. Since many tudor-domain-containing proteins localize to the nuage, we first co-stained with Vasa to determine if Tdrd5l localizes to the nuage. Our data showed that while Tdrd5l granules often closely associate with Vasa-positive nuage, they do not overlap in males (Figure 2.11). In addition, we noticed that about 50% of the 236 of "hollow" Tdrd5l granules assayed are in the cytoplasm while the other 50% are perinuclear near the nuage. However, 80% of all "hollow" Tdrd5l granules, whether cytoplasmic or perinuclear, are closely associated with Vasa-enriched granules. In females, we see no association between Tdrd5l granules and Vasa-enriched nuage (data not shown).


A
A'
A''
Vasa Tdrd5l
Tdrd5l
Vasa


B
association of Tdrd5l granules with nuage
% of granules per testis
100
80
60
40
20
0
perinuclear
cytoplasmic
next to vasa
overlapping with vasa
no association
Location

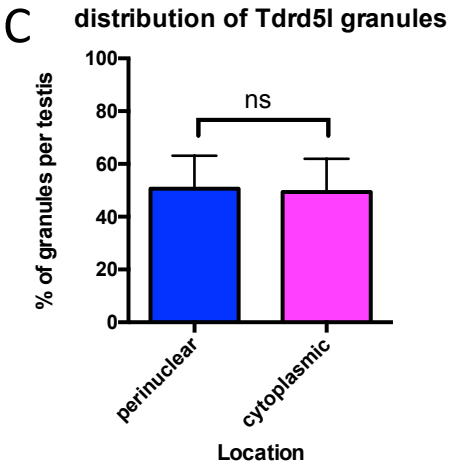

C
distribution of Tdrd5l granules
% of granules per testis
100
80
60
40
20
0
ns
perinuclear
cytoplasmic
Location

Figure 2.11: Tdrd5l granules associate with the Vasa nuage in the male germline

Testes were co-stained with Vasa and Tdrd5l to determine if Tdrd5l localized to the nuage. (A) Vasa and Tdrd5l granules appear to be closely associated in the testis but not overlapping. Quantification of these images showed that (B) the majority of Tdrd5l granules localize next to Vasa, and few overlap or have no association at all. (C) there also is no bias for Tdrd5l granules localizing perinuclear or next to the nucleus vs out in the cytoplasm. N= 236 granules

Since Tdrd5l did not localize with the nuage, we next tested whether it localized with the U body, which is another granule with Tudor-domain-containing proteins(Cauchi, Sanchez-Pulido, and Liu 2010). The U body is the location where snRNAs mature to snRNPs due to the activity of Survival Motor Neuron (SMN), also a tudor-domain-containing protein. Interestingly what we observed when overexpressing SMN:YFP was colocalization between the smaller Tdrd5l granules in the male germline and the small SMN granules (Figure 2.12A). Additionally, in both the male and female germline we observed a redistribution from granules to more cytoplasmic Tdrd5l when SMN:YFP was overexpressed. This diffuse Tdrd5l also overlapped with diffuse SMN staining (Figure 2.12 A and B).

To determine if the diffuse Tdrd5l staining we observed was due to over expression of the SMN:YFP construct, we created a CRISPR-tagged HA:SMN allele to allow us to observe localization of endogenous SMN relative to Tdrd5l. In the male germline we observed some co-localization with SMN but to a much lesser extent than when we used the over-expression construct (Figure 2.12C). This result suggested that the co-localization we observed in flies over expressing SMN is caused by this over expression. In the female germline the expression of the endogenous HA:SMN is totally different than the overexpressed YFP:SMN. When overexpressed we observed diffuse SMN in the germaria, but the endogenous construct is not expressed until much later in the female germline (Figure 2.12D). Taken together these data suggest that Tdrd5l does not normally localize to the U-body in either the male or female germline. This idea is consistent with the genetic interaction data discussed in chapter 3.

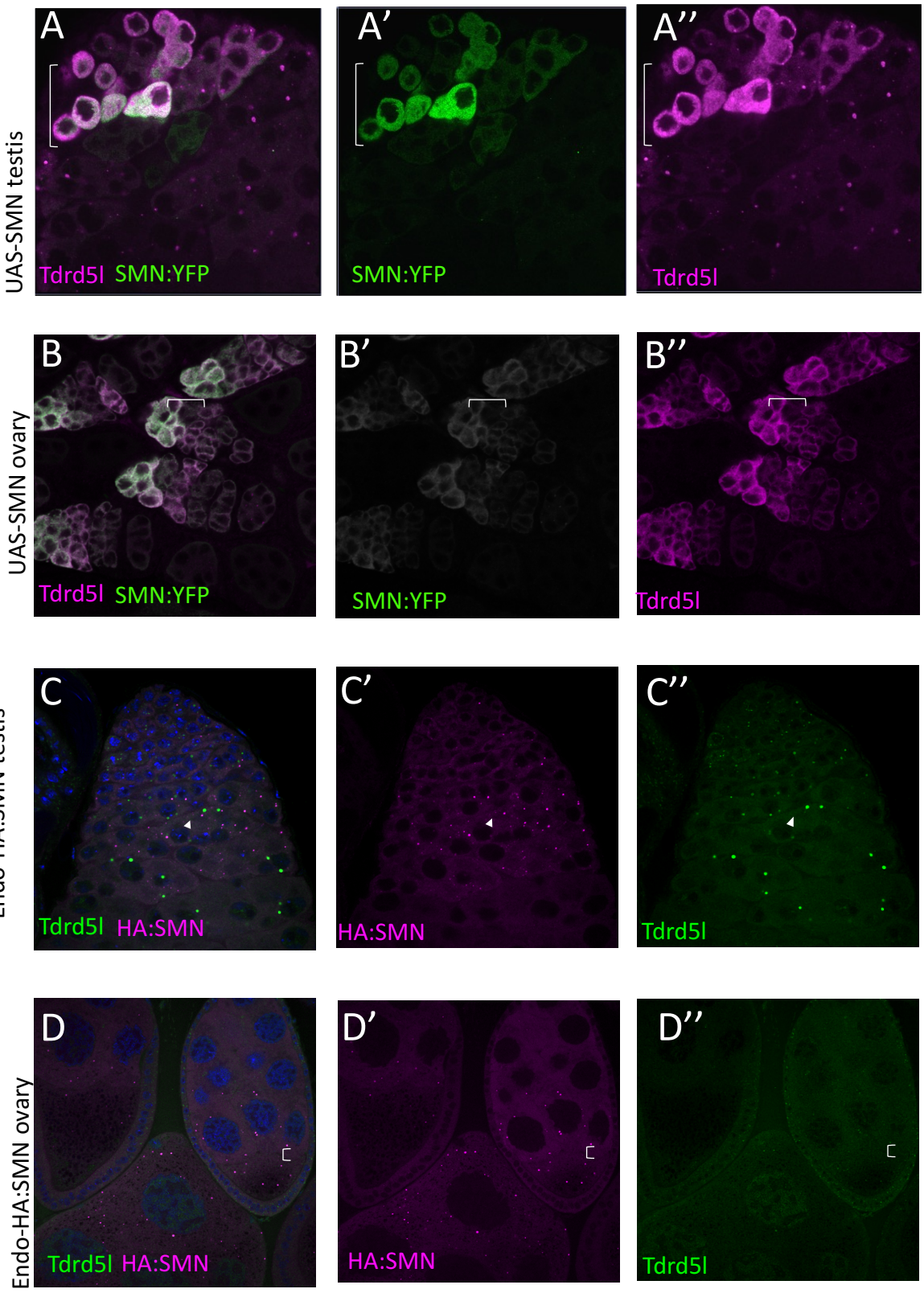

UAS-SMN testis
A
Tdrd5l SMN:YFP
A'
SMN:YFP
A''
Tdrd5l
UAS-SMN ovary
B
Tdrd5l SMN:YFP
B'
SMN:YFP
B''
Tdrd5l
Endo-HA:SMN testis
C
Tdrd5l HA:SMN
C'
HA:SMN
C''
Tdrd5l
Endo-HA:SMN ovary
D
Tdrd5l HA:SMN
D'
HA:SMN
D''
Tdrd5l

Figure 2.12 SMN associates with a small proportion of Tdrd5l granules

(A) Overexpression of SMN:YFP led to a disruption of U bodies and Tdrd5l granules in males as shown by the bracketed diffuse staining. (B) In females there was similar disruption as seen by bracketed staining in the ovaries. (C) In males expressing CRISPR-tagged SMN, there is very little overlap between U bodies marked with a white arrow and Tdrd5l granules marked by a yellow arrow. (D) In later egg chambers expressing CRISPR-tagged SMN, there are some co-localizing granules as seen inside the brackets.

Since Tdrd5l does not localize predominantly to the nuage like its homologs or to the U body where other tudor domain proteins reside, we tested whether Tdrd5l localized to the P-body, which is the most prevalent and diverse type of RNA granule. Interestingly, the P-body can dock with other granules such as the nuage and U-body; this interaction allows the transfer of RNAs between granules(Lee, Davies, and Liu 2009; L. Liu et al. 2011). Localization to such a granule could explain our observations of Tdrd5l being closely associated with the nuage and U body. To determine if Tdrd5l localizes to the P-body, we first co-stained with Decapping protein 1 (Dcp1), which is the hallmark of nearly all populations of P-bodies (Sheth and Parker 2003). Tdrd5l colocalized with Dcp1 in the male germline(Fig 2.13), and as the granules became larger in the later germline, we observed multiple Dcp1-stained P-bodies co-localizing with the periphery of the large Tdrd5l granules (Fig 2.13).

In addition to staining for Dcp1, we also co-stained for Tdrd5l and Me31b, another P-body marker. In this case we observed that the smaller Tdrd5l granules in the early germline partially colocalized with Me31b, while the later granules appeared to not co-localize with Me31b (Fig 2.14). While these data together with the Dcp1 data at first suggest that Tdrd5l localizes to the P-body, the large Tdrd5l granules are actually 3-4 times larger in diameter than P-bodies. Overall, these data suggest that Tdrd5l might localize to a previously uncharacterized RNA granule.

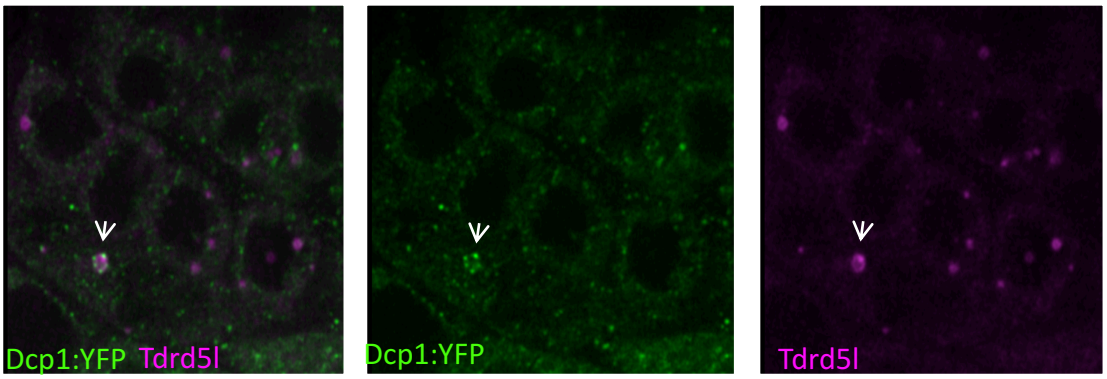


Figure 2.13 Dcp1 localizes with the periphery of Tdrd5l granules

Dcp1 positive granules in green were seen localized to the periphery of Tdrd5l granules shown in magenta

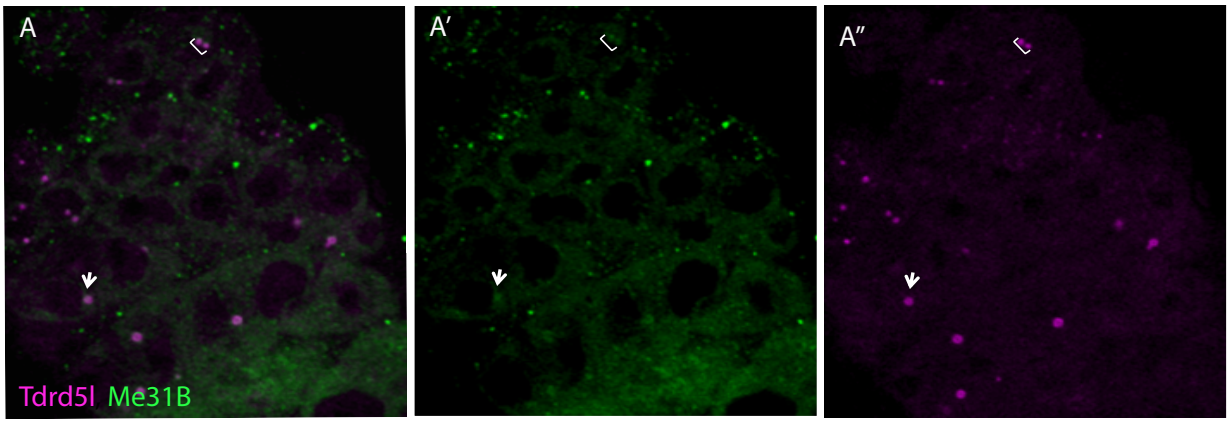


Figure 2.14 Some Tdrd5l granules localize with Me31b

Me31b granules marked in green can be seen localizing with some Tdrd5l granules in magenta.

Discussion

The work outlined in this chapter sought to determine what RNA granule Tdrd5l occupies. After testing to compare the localization and expression pattern of Tdrd5l with known granule components, it appears that Tdrd5l localizes to a novel, previously uncharacterized RNA granule. While this granule appears to be new, our data suggest that it may share some characteristics and protein components with other known granules, and further investigation might determine the Tdrd5l granule to be a sub-population of an already known granule such as the nuage or P-body due to its close association with factors found in these granules. While this possibility is interesting, our observations suggest that Tdrd5l granules themselves could be at least two different types of granule or have different functions in different cells since smaller granules seem to localize with SMN, while larger granules associate with Dcp1.

Both homologs of Tdrd5l, *Drosophila* Tej and mouse Tdrd5, localize to the nuage(Patil and Kai 2010; Yabuta et al. 2011, 5). Thus, we first expected that the nuage might also be the granule Tdrd5l occupies. While Tdrd5l did not overlap with the Vasa-positive nuage, the two granules were closely associated, and our data cannot rule out the possibility that Tdrd5l could occupy a Vasa-negative sub compartments of the nuage. In the male germline of mammals there is another granule called the chromatoid body, which associates with the nuage in a similar manner to Tdrd5l. Thus, one possibility to be tested is if the Tdrd5l granules could be the fly version of the chromatoid body, which has yet to be identified.

The major RNA granule found in all cells is the P-body and is the site of general post-transcriptional gene regulation (Sheth and Parker 2003). Different populations of P-bodies can exist based on what their function is at that moment (Tatosyan, Ustyantsev, and Kramerov 2020). The hallmark of all populations of P-bodies is Dcp1 (Parker and Sheth 2007). My data showed that Dcp1 granules associate with Tdrd5l granules, but the Tdrd5l granules are much larger and thus not likely to be P-bodies.

Interestingly, little previous data exist documenting SMN expression in the male germline, and it had been debated in the field if the U-body exists in these cells since the evidence for this structure had been shown using a UAS construct (this study). Most research involving SMN has been conducted in the nervous system since mutation of SMN causes Spinal muscular atrophy (SMA) in humans. However, data in mammals show that (SMA) model male mice also have fertility defects suggesting a role for SMN in the gonad. Our endogenous CRISPR-tagged SMN allele allowed us to determine that there is in fact SMN expression in the testis. Further study is needed to test whether the granules it localizes to are U-bodies, which are the known sites of SMN localization. These testis SMN granules are of particular interest to this study since they mark a sub population of smaller Tdrd5l granules in the testis. Further exploration of these granules could not only shed light on Tdrd5l but possibly uncover new functions for SMN if these granules are not U-bodies. SMN has previously been shown to be expressed in the female germline (Lee, Davies, and Liu 2009), and our CRISPR-tagged construct recapitulates this expression pattern. Interestingly the way in which Tdrd5l associates in the female germline offers another piece of evidence in support of the idea that Tdrd5l

might function differently in the two sexes. This hypothesis needs further exploration. Some interesting possibilities are that Tdrd5l granules could have different protein make ups between the sexes or even between cell types within a sex.

An aspect of RNA granule biology that is evident in the literature is that all of the granules we described in this study interact with each other in some way (J.-L. Liu and Gall 2007; Ketting 2011). With that in mind, if Tdrd5l granules are a previously uncharacterized granule, it would not be surprising for the Tdrd5l granules to associate with other known RNA granules. This possibility could provide a complex mechanism for careful RNA regulation, where RNAs are stored or modified in one granule and then transferred to another for further regulation or transport.

Chapter 3: Tdrd5l promotes germline differentiation through post-transcriptional gene regulation

Introduction

Tudor-domain-containing proteins have well documented functions in many if not all post-transcriptional regulatory pathways involving mRNAs and noncoding RNAs as well (Lee, Davies, and Liu 2009; Chuma et al. 2006; L. Liu et al. 2011). Additionally, tudor-domain-containing protein expression is enriched in the germline, and these proteins tend to have a slightly larger tudor domain than the minimal annotated domain. The most well studied pathway involving tudor-domain-containing proteins in the germline is that of piRNA biogenesis and transposon regulation. From flies to humans, however, that process is not the only post-transcriptional gene regulation pathway active in the germline that heavily relies on RNA regulation for development.

In the male germline, chromatin becomes tightly condensed to create aerodynamically robust sperm heads. To mediate these changes, mRNAs are heavily post transcriptionally regulated to alter when in development they will be translated.

The process is as follows. The germline stem cells undergo 4 rounds of mitotic divisions before the onset of meiosis, at which point transcription shuts off. During this time mRNAs required for translation following the completion of meiosis are transcribed and silenced until needed later. The developmental processes before and after meiosis in the male germline are dramatically different. Prior to meiosis, the goal is for the cells to grow and divide, while following meiosis the specialized process of spermiogenesis occurs and requires a vastly different proteome than mitotic germ cells. Post-transcriptional gene regulation ensures these transcripts are not translated too early and that they survive through the process of meiosis(White-Cooper et al., 1998). One

classically studied gene that are regulated in such a way is *don juan* which is transcribed in spermatocytes but the protein is present in elongating spermatids. *Don* juan transcription is regulated by the tTAF cannonball prior to the onset of meiosis (Hempel et al. 2006). Interestingly, Tdrd5l is expressed in these mitotic germ cells and localizes to large granules in the spermatocytes where these RNAs are most heavily transcribed.

Once an mRNA is transcribed in the nucleus, it is not immediately translated at that time or place. Organisms from bacteria to humans have evolved numerous post-transcriptional gene regulatory pathways that determine when and where an mRNA is translated and when it is degraded. This control allows for the removal of aberrant RNAs, localized translation, and fine tuning of developmental timing (Durand et al. 2007)(J. Smith et al. 2016).

All eukaryotic mRNAs contain a 5’ cap and a 3’ polyA tail to stabilize the transcript, and to enhance translation. One way to activate or repress translation is by the lengthening or shortening the polyA tail since translation efficiency correlates with polyA length(Norvell et al. 2015; Moore 2005). Removal of the polyA tail can trigger mRNA degradation similar to the removal of the 5’ cap, but it can also be used to temporarily repress translation until a later time.

In addition to the cap and tail, eukaryotic mRNAs have 5’UTRs and 3’ UTRs that can be targeted by small RNAs and RNA-binding proteins to regulate RNA stability and translation. Often small RNAs such as miRNAs, siRNAs, and piRNAs are loaded into an RNA-induced silencing complex (RISC) that targets the 3’UTR of an mRNA. These complexes can then recruit other proteins such as deadenylases and decapping

proteins(Fabian et al. 2011, 182). Further, adaptor proteins for mRNA transport can also bind directly to the 3'UTR.

In addition to the general mRNA decay pathways, there are some more specialized decay pathways such as nonsense mediated decay (NMD) which targets mRNAs with early stop codons to the processing body (p-body) for degradation(Durand et al. 2007). Others can detect stalled ribosomes an

One tissue where post-transcriptional gene regulation is extensively utilized is the germline. This type of RNA regulation is vital to the function and development of both the male and female germline of all species from flies to mammals. In the male germline transcription shuts off at the onset of meiosis, but a set of specialized proteins are needed following meiosis to complete spermiogenesis(White-Cooper et al., 1998.). To get around the problem of transcription shutting down so early in the germline, RNAs that are needed for spermiogenesis are transcribed in the early germline and translationally silenced until the completion of meiosis. Similarly, in the female germline, the specialized process of maternal RNA deposition relies almost entirely on post-transcriptional gene regulation. mRNAs to be deposited are highly transcribed in polyploid nurse cells and translationally repressed until they reach their proper location in the oocyte. (Wilsch-Bräuninger, Schwarz, and Nüsslein-Volhard 1997).

Prior work on *Tudor-domain-containing protein 5-like (Tdrd5l)* demonstrated that it is expressed specifically in the germline and that it localizes to cytoplasmic punctae that resemble RNA granules (Primus et al. 2019). This work also demonstrated that Tdrd5l is more highly expressed in males where it promotes male identity in the

germline. Close examination of Tdrd5l expression in the male and female germlines, described in chapter 2, shows that Tdrd5l is expressed in males starting in the germline stem cells (GSCs), but in females it is absent from the GSCs because Sex lethal (SXL) represses its translation(Primus et al. 2019). In females Tdrd5l protein can first be detected in the cystoblasts, one cell division after the GSCs. This expression pattern has led us to hypothesize that Tdrd5l promotes male identity in the germline stem cells but might possess additional functions during differentiation of the germline in both sexes.

Tdrd5l is most closely related to two other important male-biased tudor-domain-containing proteins. These proteins are TDRD5 in mammals and the true TDRD5 homolog in flies, Tejas (Tej). Both TDRD5 and Tej function in retrotransposon regulation in the male germline, and TDRD5 has an additional function in post-transcriptionally regulating non-repetitive transcripts such as normal protein coding mRNAs(Patil and Kai 2010; Yabuta et al. 2011, 5). Both Tej and TDRD5 contain a C-terminal tudor domain similar to Tdrd5l, but they also contain an N-Terminal LOTUS domain, which Tdrd5l lacks. Currently there are no predicted domains in the N- terminal half of the Tdrd5l protein, but our preliminary data from tagged proteins suggest that part of the protein could be important for localization and function. These structural differences could explain our hypothesis for how the function of TDRD5 in mammals may have diverged into two separate proteins, Tdrd5l and Tej, in flies.

Methods and Materials

mRNA sequencing

For testis mRNA sequencing, RNA was isolated using phenol chloroform extraction followed by polyA selection from 3 samples of control dissected testes and 3 samples of *tdrd5l*^{m4} dissected testes. Libraries were constructed using the protocol from (Zhang et al. 2012). 200bp paired-end sequencing was conducted on all 6 samples at the Hopkins core facility using the Illumina Miseq sequencer. Data analysis was conducted first by mapping reads to the *Drosophila* genome using Tophat (D. Kim et al. 2013). Differential gene expression analysis was conducted using cuffdiff (Trapnell et al. 2012). Genes were considered significantly differential by setting the P-value threshold at $P < 0.05$.

For ovary mRNA sequencing 3 total RNA samples of dissected ovaries from control flies and 3 total RNA samples of dissected ovaries from *tdrd5l*m4 were prepped using the Zymogen RNA micro prep kit. polyA selection, library prep, and sequencing were conducted by the Hopkins sequencing core facility. Data analysis was conducted using the same method as with males and a second time doing differential gene expression analysis using the DEseq2 R package (Love, Huber, and Anders 2014). Gene classes were considered to be enriched based on if that function was overrepresented in the list of differentially expressed genes.

Differential expression analysis for all transposons in the *Drosophila* genome was conducted using a custom pipeline written by Leif Brenner in the Oliver lab at NIH.

Genetic interaction screen

Stocks used for genetic interaction assays were +;+;*nos-GAL4, tdrd5l*^{Q5}*;; nos-GAL4, and UAS-RNAi* to genes of interest (stocks listed in appendix 1). Crosses were set up between the control nos>gal4 to RNAi and *tdrd5lQ5* ;; nos>gal4 to RNAi. All RNAi lines were crossed between both the control GAL4 and the GAL4 stock in *Tdrd5l* mutant background. Crosses were incubated at 29^{o}C and progeny from each cross were aged at 29^{o}C for either 5 days or 10days. Control RNAi progeny and *tdrd5l*-mutant RNAi progeny were dissected and stained on the same day. Gonads were imaged and compared for morphological defects. Progeny from these crosses were also aged with OregonR flies of the opposite sex to test for sterility. If there was a change in fertility or major morphological difference between the control RNAi progeny and *tdrd5l* mutant RNAi progeny in at least 50% of imaged gonads, then the gene that was knocked down was said to genetically interact with *tdrd5l*.

Granule disruption assay

Granule disruption assay was done using a similar cross and imaging scheme to the genetic interaction assays. Stocks used for females in the crosses were *nos*-GAL4; HA:Tdrd5l. For males the stocks used were mcherry RNAi for controls and RNAi against a gene of interest for the experimental crosses. Once gonads were imaged (15 gonads per genotype) the number of granules labeled by HA were quantified by counting the larger granules in males and total granules in females in the mcherry RNAi progeny and compared to the number of HA positive granules in experimental RNAi progeny.

Fly stocks and husbandry

All fly stocks were obtained from the Bloomington stock center unless otherwise noted. Orb RNAi (43143). *tdrd5l* alleles were created in our lab using CRIPSR Cas9 (Gratz et al. 2015). Both alleles used in this chapter have an early frameshift mutation resulting in no Tdrd5l protein. To create trans heterozygous *tdrd5l* mutants, *tdrd5lm4* flies were crossed to *tdrd5lq5* flies and their progeny were aged for 7 days prior to dissecting for FISH or immunostaining. The stock used to create *Tdrd5l* mutants were used as the wildtype control for experiments with trans heterozygous mutants.

Immunostaining

Dissected gonads were fixed in 4% formaldehyde in PBTx for 20min at room temperature while rotating. Following fixation, the tissue was rinsed 2x in PBTx and washed 2x for 10min in PBTx. Washed tissue was blocked in BBTX plus NGS for 30min. tissue was incubated in primary antibodies overnight while rocking at 4oC in BBTX plus NGS. Antibodies used were rabbit anti-Vasa (1:10,000, Ruth Lehman), mouse anti-Grk (1:40, Developmental studies hybridoma bank (DSHB)), mouse anti-Orb (1:20, DSHB), mouse anti-dFMR1 (1:100, DSHB) rabbit anti-Osk (1:2000, Anne Ephrussi). Samples were rinsed 2x in PBTx and washed 2x for 10min in PBTx. Samples were then stained in secondary antibody, goat IgG (Invitrogen) overnight at 4oC while rocking. Samples were then incubated in DAPI for 10min while rocking at room temperature and mounted in DABCO mounting media. Confocal microscopy was conducted using a Zeiss LSM 700. Images were processed using Zen and Fiji software.

Western Blotting

20 pairs of ovaries each were dissected from control and *tdrd5l* trans-heterozygous mutant flies and lysed in Radioimmunoprecipitation assay (RIPA) buffer plus complete mini protease inhibitor (sigma) using a pestle on ice. Following lysis, DNase treatment was conducted for 20min. Samples were spun down to remove cellular debris at 4oC for 15min. Supernatant from the spin was mixed with Lithium dodecyl sulfate (LDS) loading buffer (thermo fisher)and boiled for 10min to linearize proteins. The boiled samples were run on 4-12% SDS PAGE gels and then transferred to a membrane nu page transfer buffer (Thermo fisher) and Millipore membrane. Following transfer, membranes were blocked for 30min in 10% milk at room temperature while rocking. Membranes were incubated with primary antibodies in milk overnight at 4oC while rocking. Concentrations used were rabbit anti Vasa (1:10,000, Ruth Lehman), mouse anti Grk (1:400, DSHB), rat anti HA (1: 1000, Roche), mouse anti Orb (1:1000, DSHB). Primary antibodies were removed and the membranes were rinsed 2x in Tris buffered saline + 0.1% tween (TBTx) and washed 2x in TBTx for 10min. Membranes were incubated in HRP-conjugated secondary (1:2000, Cell Signaling) for 1hr at room temperature. Secondary antibodies were washed off following the same method as for primaries followed by a quick wash in PBS. HRP signal was then detected using the prosignal dura kit (genesse).

Results

Tdrd5l genetically interacts with post-transcriptional regulatory genes

To elucidate the molecular mechanism behind how Tdrd5l promotes proper germline development in both males and females, I conducted a directed genetic interaction screen. Since previously our lab demonstrated that Tdrd5l localizes to cytoplasmic granules that were likely to be a type of RNA granule, we included all genes involved in known post transcriptional gene regulatory pathways as well as important genes whose products are found in known RNA granules. Based on the cells in which we saw Tdrd5l expressed, we also included some important germline development genes that are expressed or repressed in those cells.

To conduct the screen, I knocked down individual genes specifically in the germline using the *nos-GAL4* and *UAS-RNAi*. I conducted these each knockdown either in wild-type animals, or in animals containing the *Tdrd5lq5* null allele, (*Tdrd5l-/+* females or *Tdrd5l-/Y* males) to determine if loss of *Tdrd5l* altered the RNAi phenotype. 20 Gonad pairs were dissected each from both male and female progeny of each genotype and stained to image morphological changes. For genes that exhibited an alteration of gonad morphology upon loss of *Tdrd5l*, we tested to see if the genetic interactions resulted in change in fertility. We quantified fertility after crossing the progeny of the RNAi cross to wildtype flies of the opposite sex as either sterile or fertile. Results of the complete screen can be found in table appendix 1. In addition to those RNA-regulatory genes, we also found interactions with some key germline development genes such as *bgcn* that need to be followed up on by a future member of the lab.

The first interaction we found was between *tdrd5l* and *twin,* which encodes a deadenylase responsible for removing mRNA polyA tails; Twin is the homolog of mammalian CCR4 of the CCR4-NOT complex. Thus, a *twin* knockdown should cause a cell to lose the ability to repress mRNA translation via shortening of their polyA tails. Under the conditions used, *twin* RNAi alone caused no noticeable morphological defects in both males and females. However, when *twin* RNAi was conducted in the germline of *tdrd5l-/y* males, the testes atrophied and the flies were completely sterile (Fig3.1A-B). When *twin* RNAi was conducted in the germline of *tdrd5l-/+* females, their ovaries had phenotypes ranging from tumors to germline loss Of the 20 ovaries quantified roughly 75 percent had tumors and the other 25% has germline loss. These flies were also completely sterile, similar to the males (Fig3.1C-D; N= 20 gonads per genotype). Thus, *twin* and *tdrd5l* exhibit a strong genetic interaction, in particular since *twin* RNAi or *Tdrd5l-/+* females have no phenotype on their own, but in combination they are completely sterile. This interaction suggests that Tdrd5l might function in the same or a parallel pathway with Twin.

Another genetic interaction that suggests a role for *tdrd5l* in a post-transcriptional gene regulatory process was the interaction with *dcp1*. Dcp1 is part of the decapping complex that removes the 5' cap from mRNAs to trigger mRNA degradation. Dcp1 is the co-factor to Dcp2 in the mRNA decapping complex and is required for efficient cap cleavage by Dcp2. What made this genetic interaction different from the *twin* interaction is that it was only present in females. There was no difference in phenotype between *dcp1* RNAi conducted in a wildtype background or a

tdrd5l-mutant background in males (Fig3.2A-B). However, in females, the genetic interaction was similar to the interaction with *twin*. There was no phenotype when *dcp1* RNAi was conducted in a wildtype germline, but knockdown in a *tdrd5l-/+* germline resulted in tumorous ovaries and germline loss as well as complete sterility (Fig3.2C-D). These results suggested that, while Tdrd5l might function in post-transcriptional gene regulation in both the male and female germline, the exact mechanism might be different between the two sexes. Other possible explanation could be that the proteome of the Tdrd5l granules could be different between the sexes, or the interacting genes may function differently between the sexes.

One other genetic interaction with a post-transcriptional gene regulatory protein also resulted in a sex-specific interaction. This interaction was with *gawky(gw).* Gw is the homolog of GW182, which binds to AGO proteins in a RISC complex following the binding of RISC to the 3'UTR of an mRNA. The Gw protein contains a long tryptophan-rich tail that recruits in other complexes such as the decapping complex and the CCR4-NOT complex (Behm-Ansmant et al. 2006). Interestingly, instead of genetically interacting with *tdrd5l* in only one sex as was the case with *dcp1, Gw* genetically interacted with *tdrd5l* in both sexes but in the opposite fashion. In males *gw* RNAi in the germline of wildtype flies results in near complete germline loss (Fig:3.3A), but when *gw* RNAi is conducted in the germline of *tdrd5l* males, this phenotype is rescued, and normal germline development is restored (Fig3.3B). However, in females *gw* RNAi in the germline of wildtype flies results in no phenotype (Fig3.3C), but when conducted in *tdrd5l* mutant background, the result is tumorous ovaries and germline loss (Fig3.3D).

This difference in genetic interaction with the same gene between males and females bolsters the conclusion from the *dcp1* interaction, which suggests that while *tdrd5l* could function broadly in post-transcriptional gene regulation, the exact mechanism by which it functions may differ between the sexes.

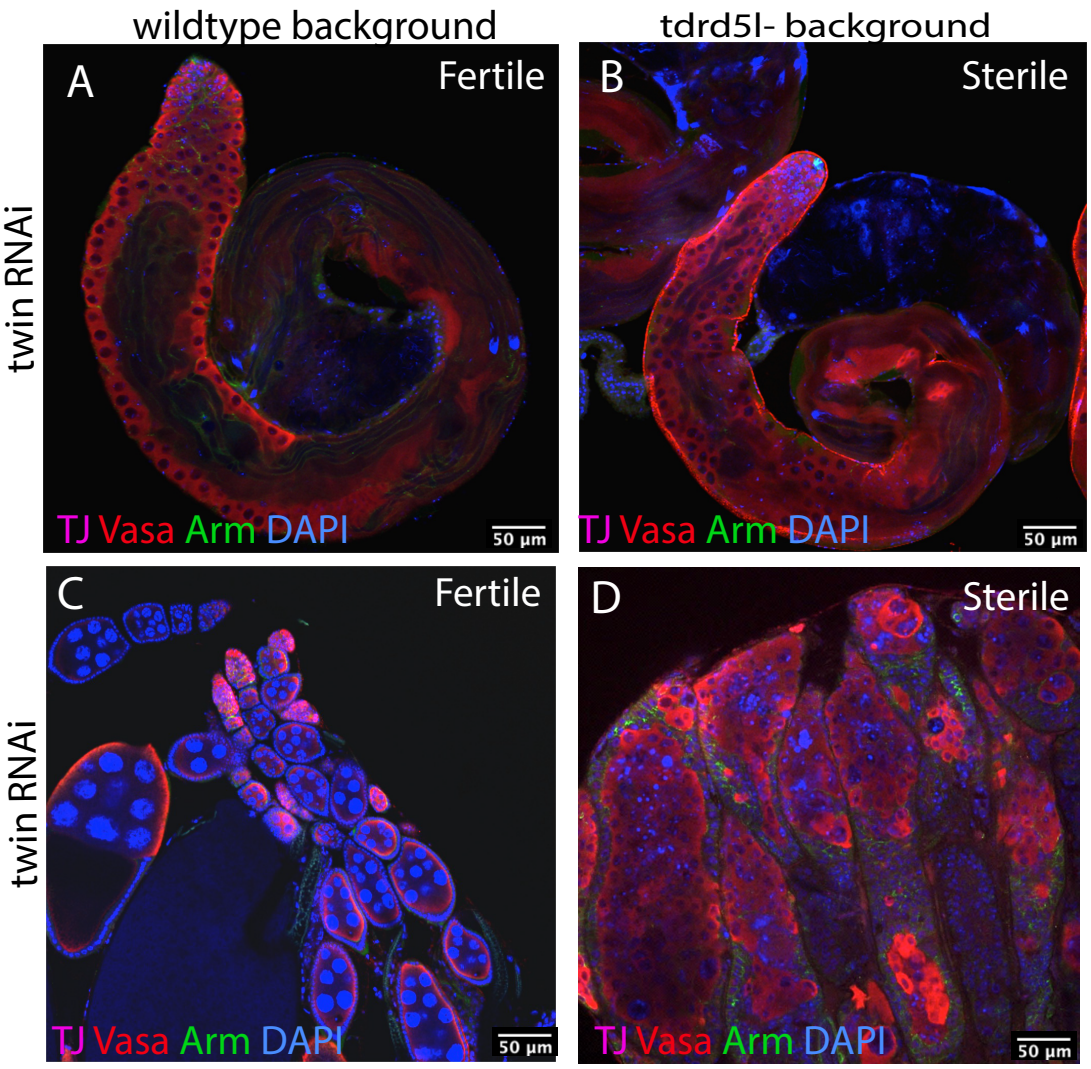

wildtype background
tdrd5l- background
twin RNAi
twin RNAi
A
Fertile
TJ Vasa Arm DAPI
50 µm
B
Sterile
TJ Vasa Arm DAPI
50 µm
C
Fertile
TJ Vasa Arm DAPI
50 µm
D
Sterile
TJ Vasa Arm DAPI
50 µm

Figure 3.1 *twin* genetically interacts with *tdrd5l*

twin RNAi in a wildtype background results in fertile males (A) and females (C) with no morphological defects. When *twin* RNAi was conducted in a *tdrd5l* mutant background, males were sterile and had atrophied testes (B). When *twin* RNAi was conducted in a *tdrd5l-/+* background in the female germline, flies had tumorous ovaries and were also sterile (D).

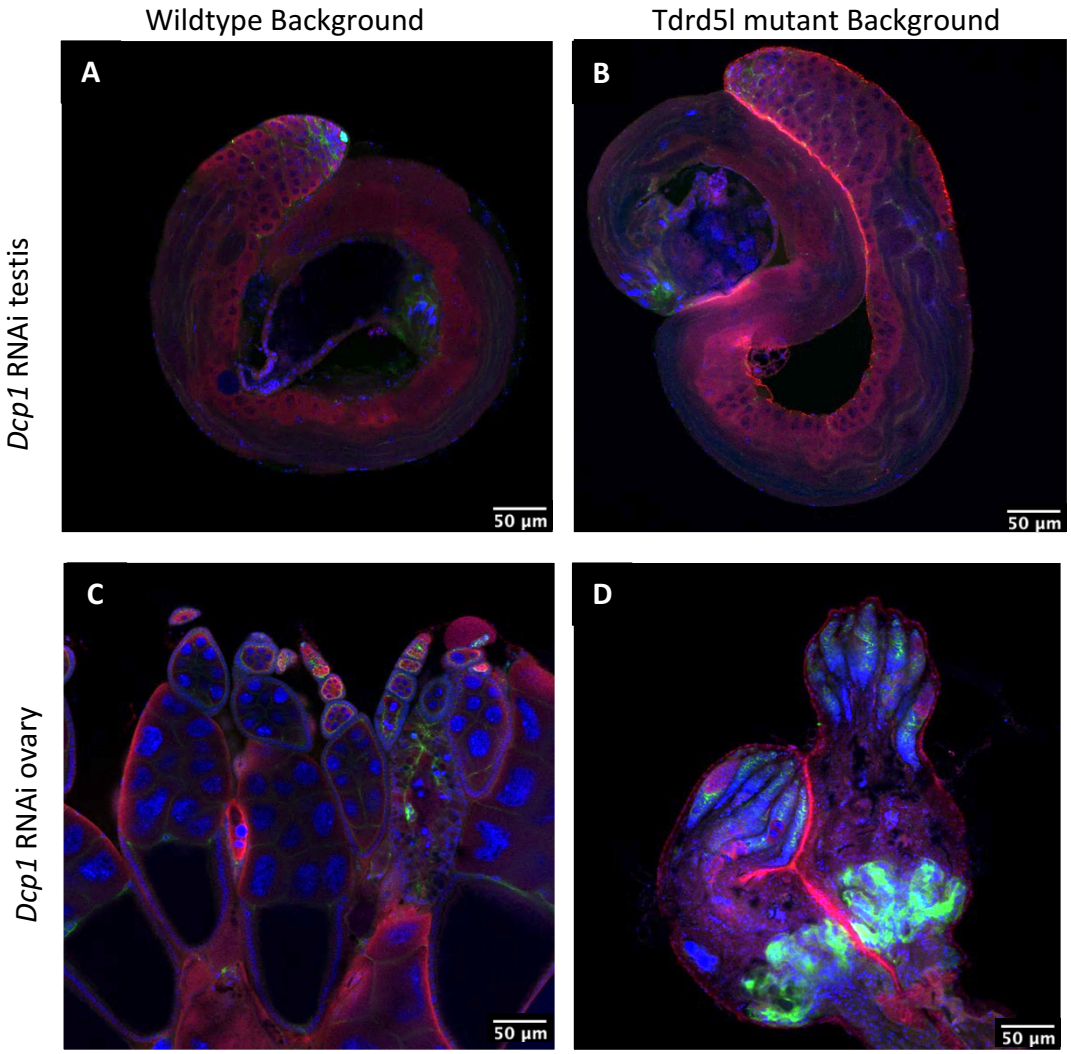


Fig 3.2 *dcp1* genetically interacts with *tdrd5l*

dcp1 RNAi conducted in a wildtype background results in fertile flies and normal gonads in both males (A) and females (C). *dcp1* RNAi conducted in a *tdrd5l* mutant background causes no defect in the male germline (B) but results in germline loss and sterility in *tdrd5l* mutant females (D).

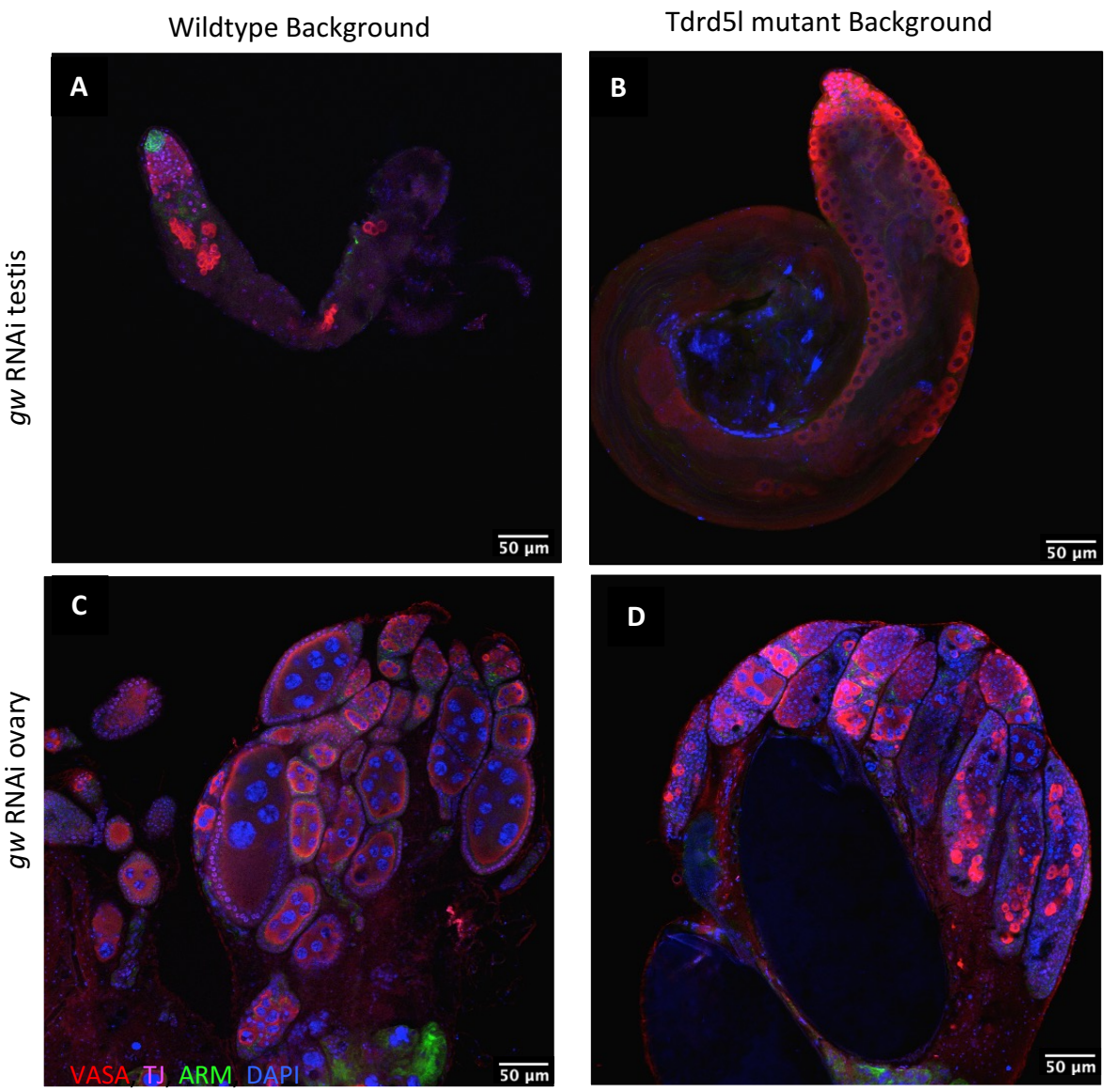

Wildtype Background
Tdrd5l mutant Background
gw RNAi testis
gw RNAi ovary
A
B
C
D
50 µm
50 µm
50 µm
50 µm
VASA TJ ARM DAPI

Fig 3.3 gw genetically interacts with *tdrd5l*

gw genetically interacts with *tdrd5l* in both males and females but in the opposite manner. *gw* RNAi in the wildtype male germline results in germline loss and sterility (A), while when conducted in *tdrd5l*-mutant males, testes develop normally (B). *gw* RNAi conducted in wildtype females resulted in no or slight germline defects (C), while when conducted in *tdrd5l*-mutant females resulted in ovarian tumors and germline loss (D).

Tdrd5l granules require small RNA pathway factors for assembly

Since the genetic interaction data suggested that *tdrd5l* may function in a post-transcriptional gene regulatory pathway, we took the next step of asking if any proteins involved in these pathways were also required for assembly of the Tdrd5l-containing granule. If this were to be the case, it would link localization of Tdrd5l to its molecular function. To answer this question, we compared the number and size of Tdrd5l granules between a control *mcherry* RNAi and RNAi for a gene of interest in the germline of both males and females. RNAi for most genes we tested did not result in any change in granule assembly. We noticed, however, a slight restriction to Tdrd5l localizing to early germ cells in the mcherry RNAi flies; this change suggests that driving any RNAi in the germline could slightly alter granule assembly. The complete list of results from this screen can be found in table 1.

Our screen uncovered 2 genes that appear to be necessary for the assembly of Tdrd5l-containing granules. These 2 proteins are Loquacious (Loqs) and Argonaute 2 (Ago2). Interestingly both of these proteins are involved in biogenesis of siRNAs and miRNAs but not piRNAs. Loqs is a double-stranded-RNA binding protein involved in the maturation of both miRNAs and siRNAs. Ago2 is the Ago protein present in the siRNA-containing RISC complex. When Loqs is knocked down in the male germline, there is a dramatic loss of granule assembly and more diffuse staining for the Tdrd5l protein when compared to the *mcherry* RNAi controls. These results suggest that the protein is still expressed but is unable to assemble into granules (Fig3.4 A-B). A similar phenotype was

seen when *loqs* was knocked down in the female germline (data not shown). When *ago2* was knocked down in the germlines of both males and females, we saw similar results to those obtained from the *loqs* knock down experiments (Fig 3.4C and not shown). Interestingly knockdown of *ago1* did not result in as dramatic of a phenotype as seen in the *ago2* knockdowns (Fig3.4D-E). Since the small RNA biogenesis pathways associated with both Loqs and Ago2 are involved with targeting mRNAs to granules for silencing, this further supported our hypothesis that Tdrd5l functions in post transcriptional gene regulation. Additionally, Since Ago1 and Ago2 function in different small RNA mediated pathways, the difference is granule disruption between knocking down these two genes could indicate what small RNA pathway is active in the granules.

Gene	**Change in granules**
AGO1	Slight decrease
AGO2	Decrease
bgcn	No change
cu	Slight decrease in females
Dcr-1	No change
eif5b	Decrease in females
gw	No change
loqs	decrease
orb	Increase in females
orb2	No change
Smn	No change
tej	No change
vasa	No change

Table 3.1: Summary of granule disruption assay

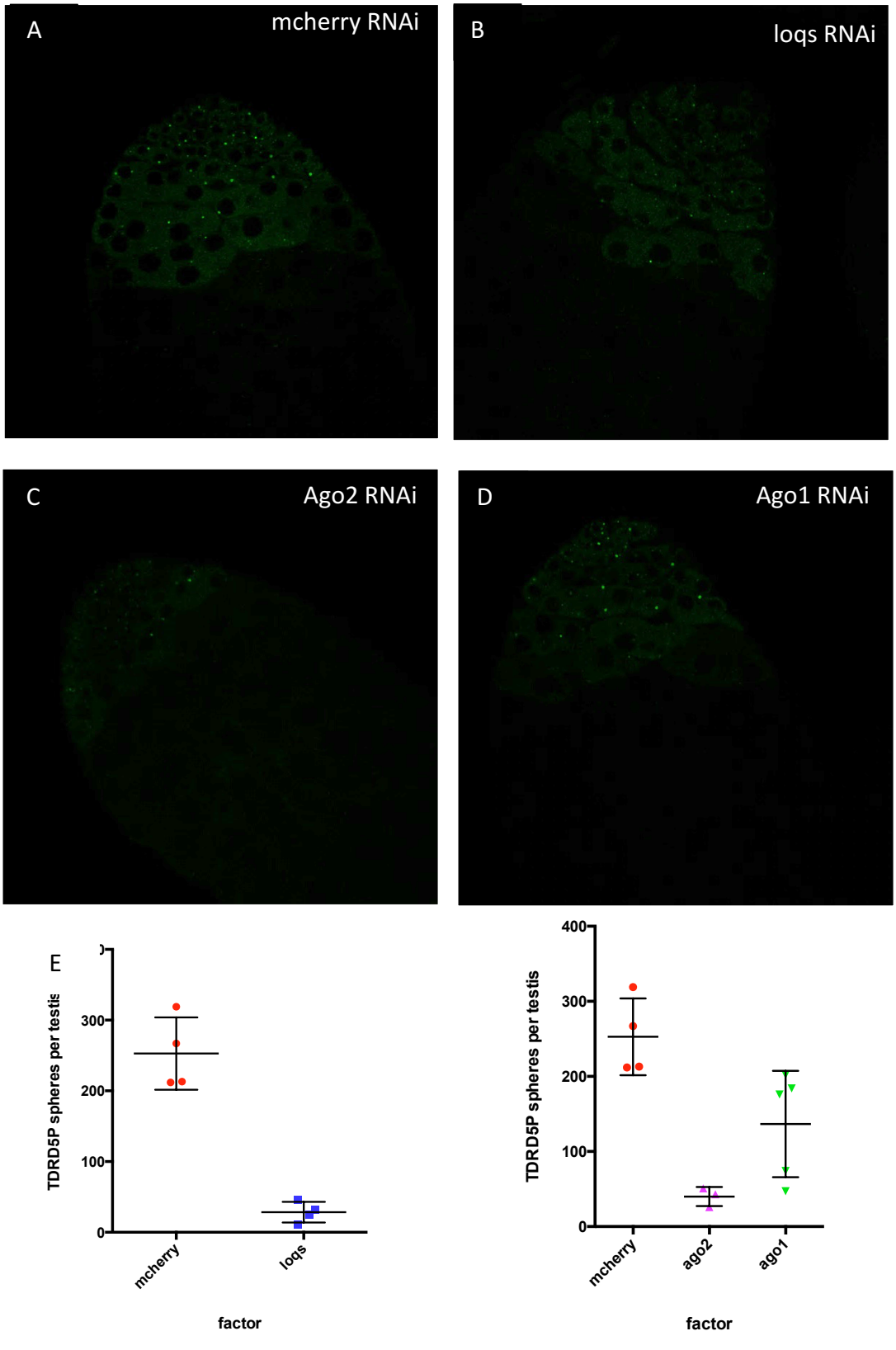

A
mcherry RNAi
B
loqs RNAi
C
Ago2 RNAi
D
Ago1 RNAi
E
TDRD5P spheres per testis
300
200
100
0
mcherry
loqs
factor
TDRD5P spheres per testis
400
300
200
100
0
mcherry
ago2
ago1
factor

Fig 3.4 Loquacious and Ago 2 are required for Tdrd5l localization to granules

When compared to the mcherry RNAi control (A), *Loqs* RNAi resulted in loss of localization of Tdrd5l to granules in the germline (B). A similar reduction was seen in Ago2 RNAi testes (C), but to a lesser extent in Ago1 RNAi testes (D). Quantification of granules per testis is graphed in (E). Changes seen in *loqs* and *ago2* are statistically significant based on $P < 0.05$ using students T test.

mRNA sequencing of *tdrd5l* mutants reveals few changes in gene expression

To identify genes whose expression levels were regulated by Tdrd5l, I conducted mRNA sequencing from testes of control flies and *tdrd5l*-mutant flies using a library construction protocol optimized for *Drosophila* testes(Zhang et al. 2012). As might be expected for a gene suspected to play a role in post-transcriptional gene regulation, very few genes had significant differences in gene expression between control and mutant flies.

There were few genes with large significant changes in expression levels in *tdrd5l* mutant ovaries. 970 genes exhibited a significant increase in expression in *Tdrd5l* mutant ovaries. Of those 970 genes, 55 genes increased by a log2-fold change greater than 2. In addition, 858 genes had a significant decrease in gene expression in *Tdrd5l* mutant ovaries. Of those 858 genes, 126 of them decreased in expression by more than a log2-fold of 2 or more (Figure 3.6). In addition, maternal RNAs tended to be enriched in this data set, and that observation is described in more detail in chapter 4.

Of the 720 genes determined to have a significant decrease as described in the methods in gene expression in *tdrd5l* mutants, only 33 had a log2-fold of 2 or greater decrease in expression. Additionally, there were no trends in terms of what types of genes displayed a decrease in expression. Nothing stood out from the data set in terms of common pathways occupied by genes that had changes in expression. Of the 290 genes determined to have a significant increase in expression in *tdrd5l* mutants, 33 of these genes had a log2-fold change of 2 or greater increase in expression (figure 3.6). Of

these 290 genes, there was an enrichment for genes predicted to be sugar transporters, immune genes, and genes containing chitin-binding domains. The gene with the highest difference in gene expression was *male specific transcript 36fb (mst36fb)*, which displayed a ~500 fold increase in transcript levels in *tdrd5l* mutants (Fig3.5). Finally, since *tdrd5l* mutants did not exhibit many large changes in gene expression, I can rule out the possibility that Tdrd5l has a wide role in RNA stability.

mst36fb was also of particular interest due to its expression pattern in the testis. Two published studies revealed that the mRNA is expressed during the 16 rounds of mitosis, but the protein isn't translated until the completion of meiosis. These studies also determined that expression of *mst36fb* is promoted by both *aly* and *can*, classes of meiotic arrest genes that are highly expressed at the same time point we see Tdrd5l localizing to larger granules in the spermatocytes. There are no known conserved domains in the Mst36fb protein, and the function remains unknown.

Figure 3.5 *mst36fb* is highly expressed in *tdrd5l* mutant testes

Sequencing of mRNA derived from testes demonstrates that *mst36fb* has a 500-fold increase in expression in *tdrd5l* mutants (purple) compared to control testes (orange).

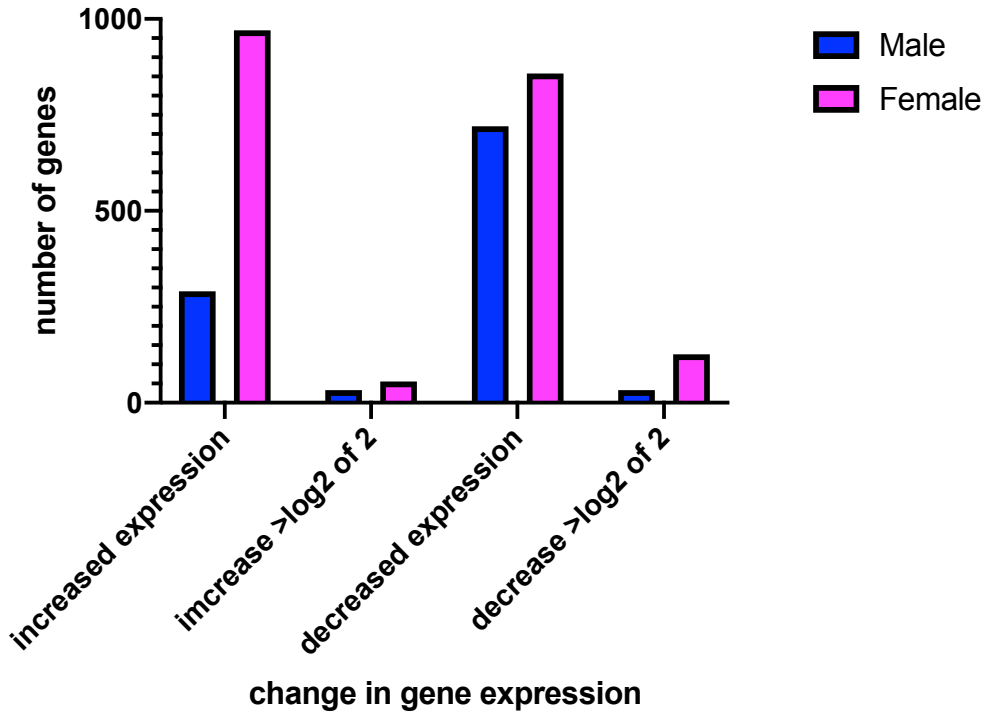


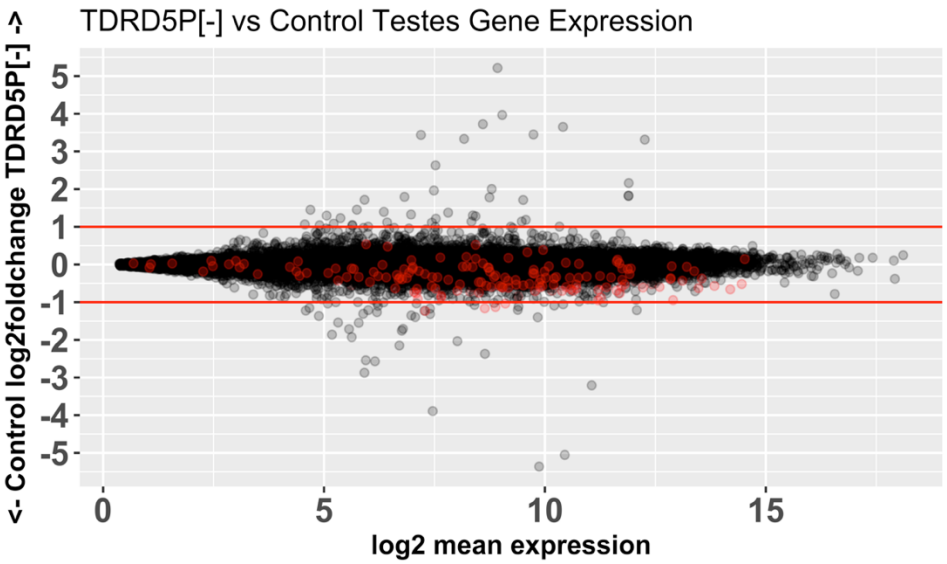
Figure 3.6 Changes in gene expression in *Tdrd5l* mutant males and females

Graph of overall trends in gene expression changes from *Tdrd5l* males and females. Shown are the overall increases and decreases, as well as how many genes were highly changed in each subset. Male data are represented by blue bars and female data are represented by magenta bars.

Tdrd5l does not regulate transposons

Since the two closest homologs to Tdrd5l, Tdrd5 and Tej, both repress transposon expression, I conducted an analysis on both the male and female RNAseq data sets to determine if *tdrd5l* also regulates transposon expression. My analysis showed that on a genome-wide scale, Tdrd5l does not repress transposons globally in the male germline (Fig 3.6a) or the female germline (Fig 3.6b), suggesting a functional divergence from its closest homologs. One interesting possibility that these data do not rule out is that Tdrd5l might still function in a small-RNA pathway such as piRNAs similar to its homologs.

A


TDRD5P[-] vs Control Testes Gene Expression
<- Control log2foldchange TDRD5P[-] ->
5
4
3
2
1
0
-1
-2
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-5
0
5
10
15
log2 mean expression

B

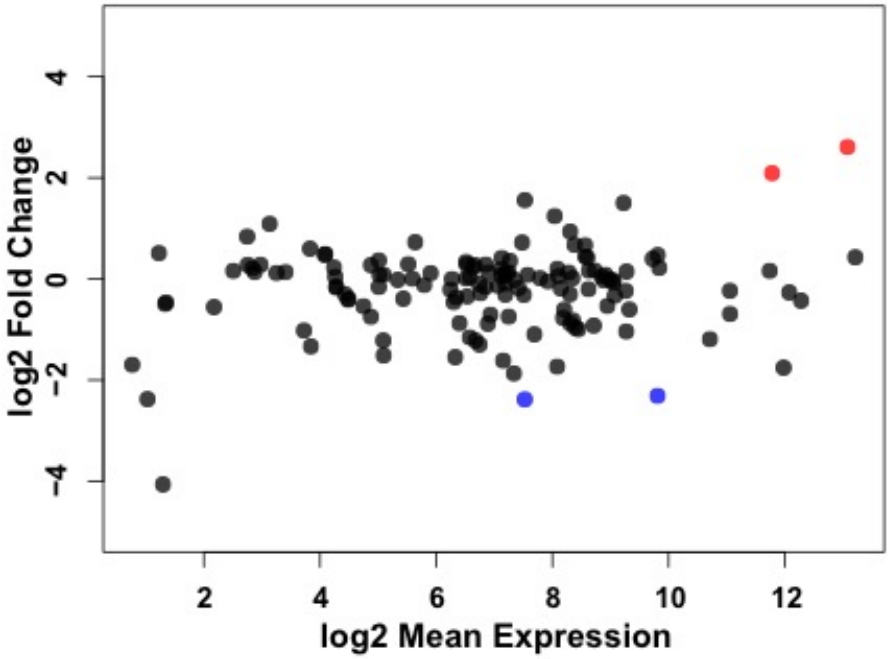

log2 Fold Change
4
2
0
−2
−4
2
4
6
8
10
12
log2 Mean Expression

Figure 3.6: Tdrd5l does not repress transposon gene expression

In the male germline (A) no transposon genes (marked in red) have a significant change in gene expression. B) a plot of all transposon genes in the female germline shows only 4 transposons have any significant changes in gene expression.

Discussion

Previous work on Tdrd5l described a male-specific function in the germline (Primus et al. 2019), however the work described here as a continuation of previous studies demonstrated a role for Tdrd5l in both the male and female germlines. Specifically, in the female germline I found that Tdrd5l regulated maternally deposited RNAs. I drew this conclusion from broad observations on RNA seq data that were reinforced with a few specific examples. Interestingly, this same RNAseq data showed that Tdrd5l also differs from its homologs in that it doesn't globally repress transposons in either sex. In females there were four transposons that had changes in gene expression, and future work could investigate their identity and mode of regulation.

The germline is a unique tissue type that relies heavily on post-transcriptional gene regulation of mRNAs. Examples of this regulation are the shutdown of transcription in the early male germline followed by translation of specific mRNAs after meiosis. Another is the specialized program of maternal RNA deposition. While these instances are broad examples, some specific maternal RNAs are regulated by their own sets of proteins(Nakamura, Sato, and Hanyu-Nakamura 2004; Clouse, Ferguson, and Schüpbach 2008). Such complexity relies on RNPs that can be unique to different mRNAs. Some of the proteins in these RNPs bind directly to the target mRNA while others are cofactors, or scaffold proteins, as is the case for many tudor domain proteins.

To identify the function of Tdrd5l I did a genetic interaction screen based off known functions of tudor-domain-containing proteins and RNA-regulation pathways in the germline. Our screen revealed that *tdrd5l* genetically interacts with *twin, dcp1,* and

gw, all of which are involved in post-transcriptional gene regulation and are known to function in the germline. Interestingly, while *tdrd5l* had a similar enhanced phenotype when *twin* was knocked down in males or females, the interactions with *gw* and *dcp1* differed based on sex. This result suggests that in addition to the sex-promoting function of Tdrd5 being sex specific, the way in which it functions in a post-transcriptional gene regulatory pathway could differ between males and females.

Lastly, given that our genetic interaction data suggested a role for Tdrd5l in post-transcriptional gene regulation, we asked whether the presence of certain factors in these pathways are necessary for Tdrd5l to be recruited to the granule it occupies. When we knocked down either Loqs or Ago2, Tdrd5l failed to localize to granules suggesting that an active mRNA silencing pathway is required for Tdrd5l to localize to granules. This result fits with years of work in the RNA granule field that demonstrates that often an RNA is required first to seed a granule, and that in many cases, particular proteins are necessary for other proteins to be recruited to a granule. Further work to identify what protein and RNA factors localize with Tdrd5l will be necessary to determine the exact mechanism used by Tdrd5l to promote germline differentiation. These experiments will also allow us to determine if Tdrd5l has a general function in post transcriptional gene regulation or if it only regulated a few specific target RNAs.

Chapter 4: Tdrd5l regulates RNA metabolism and maternally deposited RNAs

Introduction

The female germline relies heavily on post-transcriptional gene regulation during development. Unlike the male germline where all 16 cells in the final mitotic cyst enter meiosis, in females only one of the 16 cells will become an oocyte, while the other 15 cells in the cyst become polyploid nurse cells. These nurse cells function to produce the mRNAs that need to be maternally deposited into the developing oocyte. Some of these RNAs, such as *bicoid, nanos*, and *gurken*, will set up the body axes of the future embryo(F. S. Neuman-Silberberg and Schupbach 1994; Gonzalez-Reyes and Johnston 1998; Frohnhöfer and Nüsslein-Volhard 1986). Other maternally deposited RNAs encode transcription factors and other proteins needed before zygotic transcription turns on in the embryo.

Since these maternally deposited RNAs need to make it to the oocyte without being translated in the nurse cells, these mRNAs are subject to post-transcriptional silencing as they get transported to the oocyte. Once in the oocyte they often continue to be silenced until they arrive at their proper location and development progresses to the time when their protein product is needed. Blocking of translation can be accomplished via small-RNA-mediated silencing or from RNA binding proteins interacting with the untranslated regions of the mRNA (Behm-Ansmant et al. 2006; Fabian et al. 2011).

One example of how these RNAs are regulated involves the *gurken* (*grk*) mRNA. Grk protein is needed first at the posterior of the oocyte to specify the posterior follicle

cells. *grk* RNA then moves to the dorsal anterior corner of the oocyte where Grk protein is again produced, this time to specify the dorsal side. The *grk* mRNA is post-transcriptionally silenced in the nurse cells and activated by Orb protein in the posterior and dorsal anterior corner of the oocyte(Chang et al. 2001). *grk* mRNA is not translated in the nurse cells due to the absence of its activator protein Orb (Davidson et al. 2016) and due to the repressor Squid (Clouse, Ferguson, and Schüpbach 2008; Delanoue et al. 2007). The *orb* mRNA itself is maternally deposited and post-transcriptionally silenced by Cup, which interacts with translation initiation factor, and by dFMR1, which binds to the 3' UTR to repress translation(Wong and Schedl 2011; Costa et al. 2005).

In this chapter I outline my work on how Tdrd5l regulates *grk* protein expression. I chose to investigate this particular gene due to defects seen in eggs laid by *Tdrd5l* mutant females that indicated there could be defects in axis specification. Additionally, I sought to understand the mechanism by which Tdrd5l might regulate these RNAs.

Materials and Methods

Fly stocks

All fly stocks were obtained from the Bloomington stock center or from the noted sources; *twin* RNAi (32490 and 32901), *gawky RNAi* (34796), and *dcp1* RNAi (67874). *tdrd5l* mutant alleles were previously created in our lab using CRISPR/Cas9 mutagenesis (Gratz et al. 2015). The two *tdrd5l* mutant alleles used in this study were $tdrd5l^{M4}$ and $tdrd5l^{Q5}$. These alleles contain frame shift mutations in the first 20bp of the RNA and result in a protein null.

Female fecundity assays

Female fecundity assays were conducted using 5-day old *Tdrd5l[M4]/Tdrd5l[Q5]* trans-heterozygous females. Control flies used were females from the stock used to make CRISPR mutants that were crossed to OregonR males. Single-female eggs lays were done on grape juice plates with yeast paste at 25^{o}C for 24hrs. After 24hrs, flies were flipped to a new plate and total eggs as well as eggs with dorsal appendage defects were counted. Eggs were incubated at 25^{o}C for 24hrs again. Following incubation, the number of eggs hatched were counted as well as the number of unhatched eggs with dorsal appendage defects. Percentages of hatched and unhatched eggs were calculated and compared between control egg-lays and mutant egg-lays.

Immunofluorescence

Dissected gonads were fixed in 4% formaldehyde in PBTx for 20min at room temperature while rotating. Following fixation, the tissue was rinsed 2x in PBTx and washed 2x for 10min in PBTx. Washed tissue was blocked in BBTX plus NGS for 30min. Tissue was incubated in primary antibodies overnight while rocking at 4^{o}C in BBTX plus NGS. Antibodies used were rabbit anti-Vasa (1:10,000, Ruth Lehman), guinea pig anti-Traffic Jam (1: 1000, Jennifer Jemc), mouse anti-Arm (1:500, DSHB), rat anti-HA (1:100, Roche), mouse anti-Grk (1:40, (DSHB)). Samples were rinsed 2x in PBTx and washed 2x for 10min in PBTx. Samples were then stained in secondary antibody, goat IgG (Invitrogen) overnight at 4^{o}C while rocking. Samples were then incubated in DAPI for 10min while rocking at room temperature and mounted in DABCO mounting media.

Confocal microscopy was conducted using a Zeiss LSM 700 or LSM 800 with airyscan. Images were processed using Zen and Fiji software.

Fluorescence in situ hybridization

FISH was conducted on dissected ovaries from one-week-old flies according to the protocol in ((Zimmerman et al. 2013). *osk* and *grk* sense and antisense probes were transcribed in vitro from clone LD32255 for *grk* and clone LD24944 for *osk*. These probes were used at a 1:1000 dilution. TSA amplification was conducted for 1hr for both *osk* and *grk* FISH experiments.

Results

Tdrd5l is required for oogenesis in addition to spermatogenesis

Previous studies in our lab focused on understanding the function of Tdrd5l in the male germline (Primus et al. 2019). To take a broader look at potential Tdrd5l functions in the germline, I investigated morphological defects in dissected gonads of both sexes. Previous work demonstrated that *tdrd5l* mutant males had expanded DAPI-bright regions in the apical tip of the testis, a phenotype that is indicative of delayed entry into meiosis(Primus et al. 2019). I observed this effect in my experiments as well (data not shown). The published localization of Tdrd5l in the premeiotic germline taken together with published information of post-transcriptional gene regulation in this stage of germline development led me to investigate the development of the late male germline following the completion of meiosis.

Since the genes necessary for spermiogenesis are transcribed in the early germline and translated following the completion of meiosis, I investigated whether there was a phenotype in sperm maturation. Preliminary analyses revealed a potential defect in spermatid elongation, and this phenotype warranted further investigation.

Defects in spermatid elongation can be detected by staining for actin, to look at the acrosome, and caspase, which stains the elongation complex as well as the waste bags. The elongation complex in *tdrd5l* mutants appeared more elongated and narrower compared to the wildtype elongation complex (Fig 4.1 A-B).

To investigate the female germline in more detail than our previous study, we first looked for broad morphological defects in the ovary. In *tdrd5l* mutant I observed fewer late-stage egg chambers than wildtype flies. Additionally, in *tdrd5l* mutant ovaries I saw an increase in fragmented DAPI staining compared to wildtype ovaries, a phenotype that is indicative of cells undergoing cell death. (Fig 4.1 C-D). To confirm this possibility, I stained for cleaved caspase, which is used in programmed cell death (Kumar 2007). After aging females for 5days I saw increased cleaved caspase staining in *tdrd5l* mutants, supporting the hypothesis that the fragmented DAPI staining was due to an increase in cell death. (Fig 4.2 A-C))

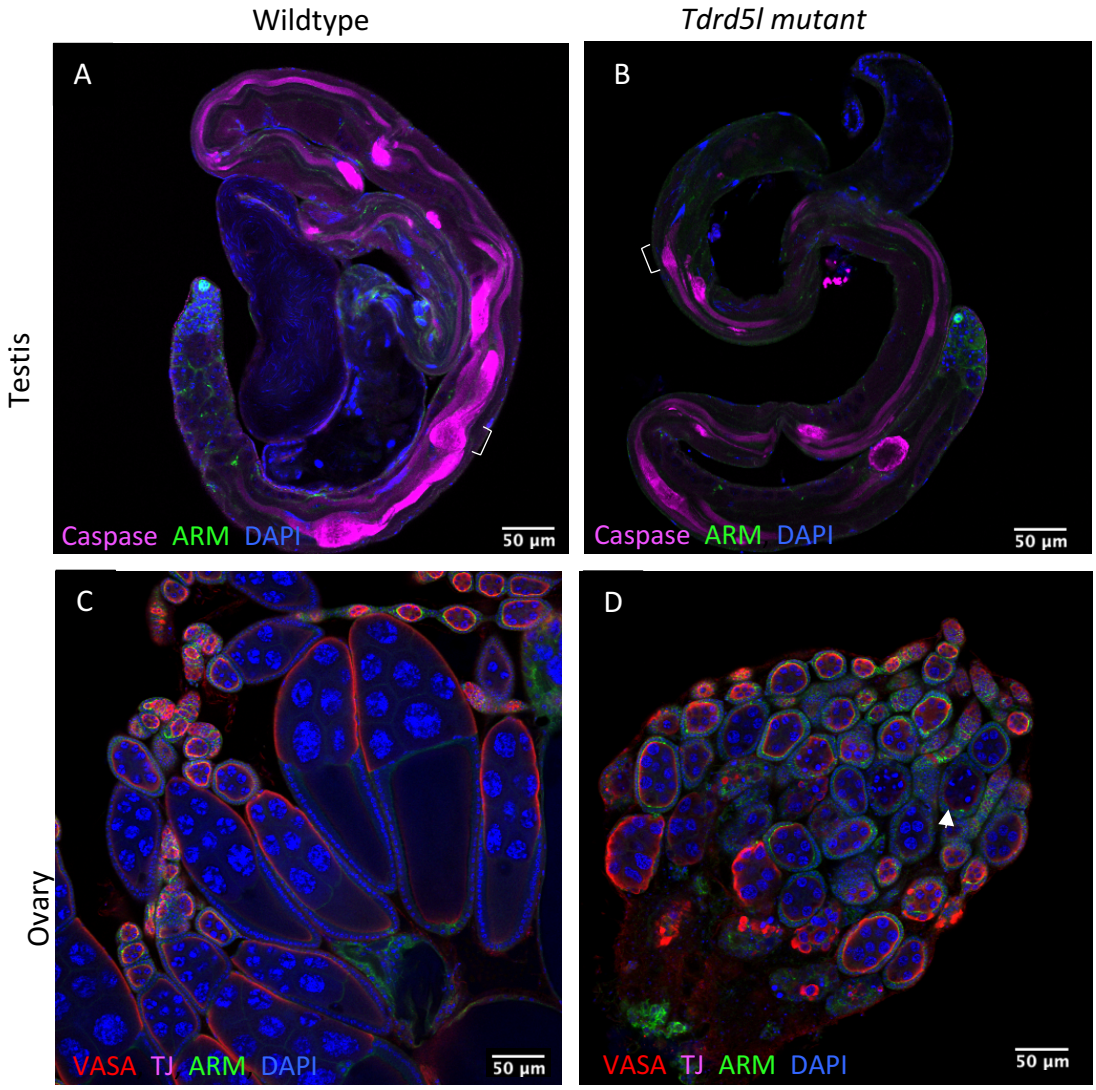

Wildtype
Tdrd5l mutant
Testis
Ovary
A
B
C
D
Caspase ARM DAPI
Caspase ARM DAPI
VASA TJ ARM DAPI
VASA TJ ARM DAPI
50 µm
50 µm
50 µm
50 µm

Figure 4.1 Tdrd5l mutants have defects in germline development

Immunostaining of wildtype (A) and *tdrd5l* mutant (B) testes shows changes in caspase staining, which reveals elongated individualization complexes in *tdrd5l* mutant males. Immunostaining of wildtype (C and E) and *tdrd5l*-mutant (D and F) ovaries shows an increase in cell death as indicated by fragmented DAPI staining (D) and increased caspase staining (F) in *tdrd5l*-mutant females.

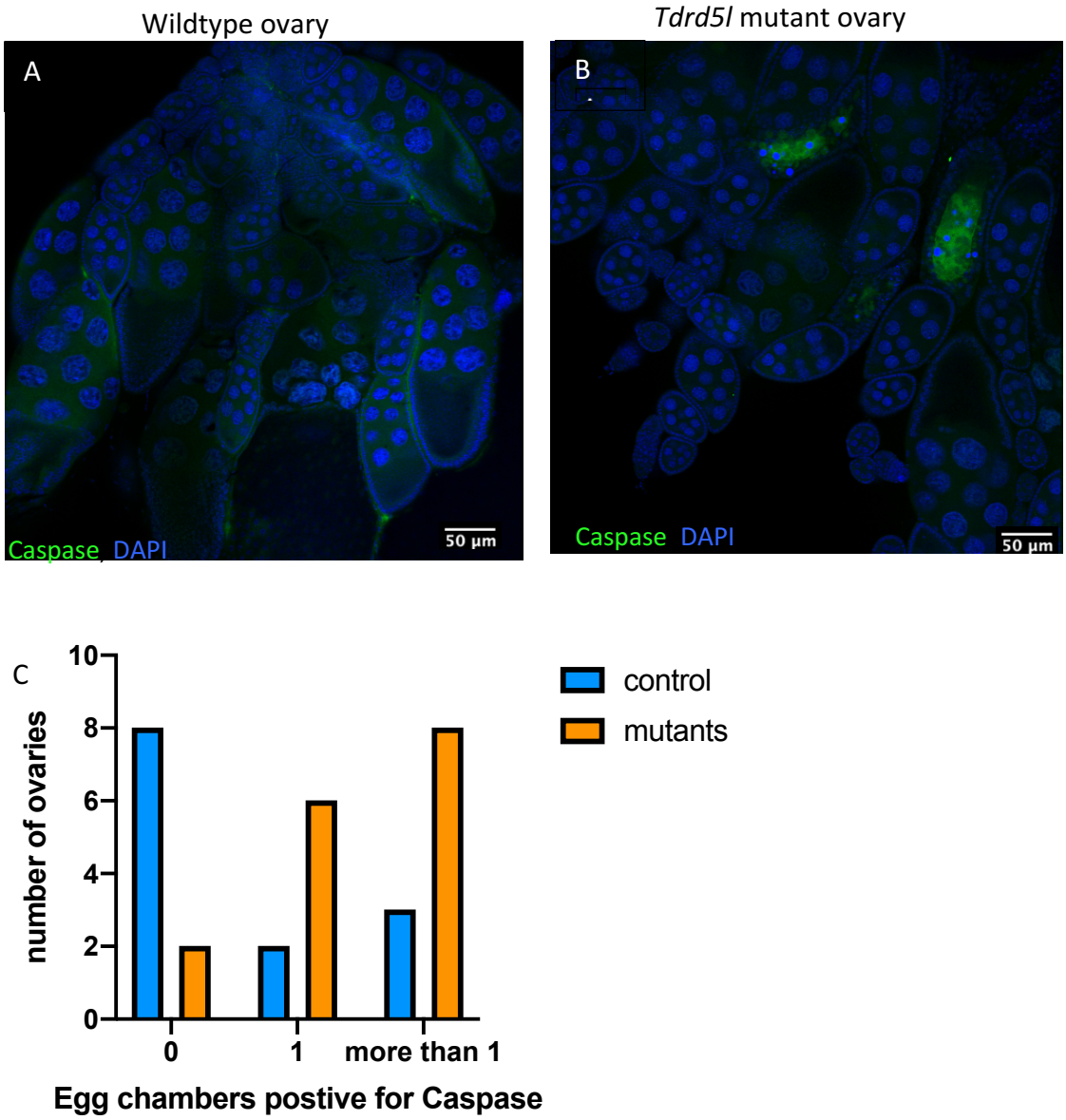


Figure 4.2: *Tdrd5l* mutants have increased cleaved caspase staining

(A) Wildtype ovaries have minimal cell death as marked by cleaved caspase staining (green). DAPI = blue. (B) *Tdrd5l* mutant ovaries have increased levels of cell death as indicated by caspase staining. (C) Quantification of cell-death phenotype differences between are significant by T test.

Tdrd5l promotes egg development

Since previous data demonstrated a loss of fecundity in males, I reasoned that reduced fecundity might occur in females as well, especially in light of the morphological defects I uncovered in the female germline. Since morphological phenotypes were not 100% penetrant, I conducted fecundity assays as single male and female matings. To minimize effects caused by background mutations, all *tdrd5l* mutant females were trans-heterozygous for two different *tdrd5l* alleles. All controls were the base stock used to create the mutant alleles.

When comparing the number of eggs laid by individual females, I found a reduction in the number of eggs laid by *tdrd5l* mutant females (Fig 4.3A). In addition to fewer eggs being laid, far fewer eggs laid by mutant females hatched after 24hrs at 25^{o}C (Fig 4.3B). These results suggested that the lack of late-stage egg chambers detected in the morphological assays could be causing fewer eggs to be laid overall by *tdrd5l* mutant females.

Due to the decreased hatch rate, I examined the embryos for phenotypes. Many of these eggs did not appear to develop properly, consistent with a significant portion of eggs laid by *tdrd5l* mutant females displaying dorsal-appendage defects, which reveal errors in dorsal-ventral patterning (Fig 4.3C) (Berg 2005; Osterfield, Berg, and Shvartsman 2017) Many but not all of these unhatched eggs laid by mutants were the eggs with dorsal appendage defects (Fig4.3D). The dorsal appendage defects seen in these eggs implicate Tdrd5l in *grk* regulation, since errors in *grk* localization or

translation result in eggs with defective dorsal appendages and faulty embryonic dorsal/ ventral patterning (F S Neuman-Silberberg and Schupbach, 1994.)

When I took a closer look at eggs with dorsal appendage defects, there was no single type of defect present. A typical wildtype dorsal appendage resembles an oar, with a long stalk and a flat paddle as seen in Fig 4.4A. I grouped the appendage defects into 3 broad categories that represent the majority of the phenotypes observed. The first class consisted of one somewhat-normal-looking appendage with the second being either absent or truncated (Fig 4.4B). The second type of dorsal appendage defect was a complete lack of an oar-like structure; instead, the two appendages developed into a crown encircling the anterior end of the egg (Fig 4.4C). The last class of defects consisted of eggs that had no dorsal appendage structure (Fig 4.4D.). As mentioned above, defects in dorsal appendage morphology indicate that the dorsal ventral axis was not being specified properly. These defects reveal that the eggs have either become dorsalized (Fig. 4.4C) or ventralized (Fig. 4.4D) and suggest that *grk* mRNA may not be regulated properly. Since the entire body plan in *Drosophila* is set up by maternally deposited RNAs, we reasoned that this phenotype warranted investigation into whether Tdrd5l regulates these RNAs.

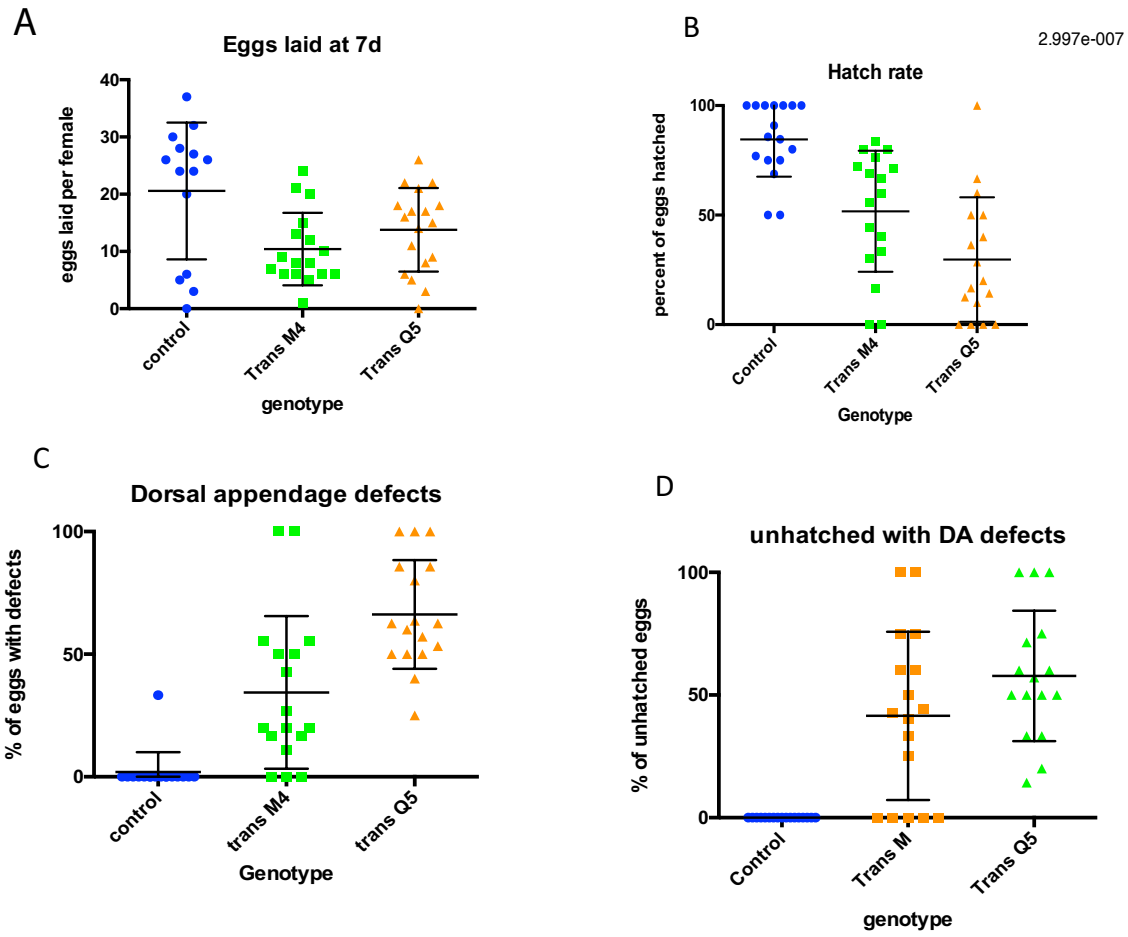


Figure 4.3 Tdrd5l mutant females have decreased fecundity and dorsal appendage defects

Single fly egg count assays show (A)*tdrd5l* mutant females lay less eggs than wild type females. Each dot represents eggs from a single female. (B) eggs laid by *tdrd5l* mutant females have a lower hatch rate than eggs laid by wildtype females. (C) Many eggs laid by *tdrd5l* have dorsal appendage defects, (D) many of these eggs account for the unhatched eggs laid by *tdrd5l* mutants

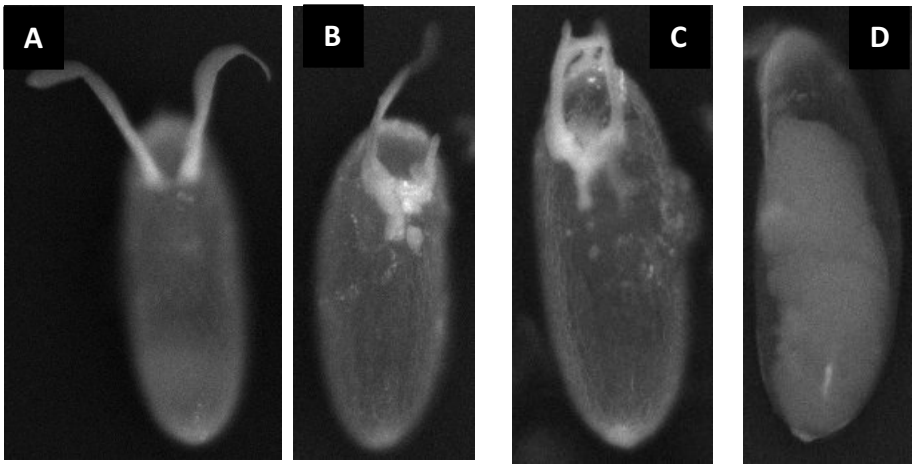


Fig 4.4 Eggs laid by *tdrd5l* mutants have dorsal appendage defects

(A) Wildtype dorsal appendages resemble two symmetric oars, each with a long stalk and a flat paddle. Eggs laid by *tdrd5l* females present a range of dorsal appendage defects. (B) an example of uneven dorsal appendages, (C) dorsal appendage material that forms a crown-like structure, or (D) no dorsal appendages.

Tdrd5l represses protein expression of Grk and Osk

Maternally deposited RNAs are highly regulated when it comes to their time and place of translation. Since our RNAseq data indicate potential regulation of maternal RNAs by Tdrd5l, showed dorsal appendage defects in *tdrd5l* mutant eggs, I asked whether there were changes in Grk protein expression in *tdrd5l* mutants. To answer this question, we immunostained control ovaries and *tdrd5l* trans-heterozygous mutants for Grk protein. As expected in control ovaries we see Grk protein localized to the dorsal anterior corner of the developing oocyte where it is secreted to the neighboring follicle cells (Fig 4.5a,b). Interestingly, in *tdrd5l* mutants, Grk was ectopically expressed in the nurse cells and surrounding follicle cells, as well as in the developing oocyte (Fig4.5 C,D and I). It should also be noted that in *tdrd5l* mutants, Grk was still localized to the proper location of the oocyte but possibly at lower levels. Since we observed proper localization in the oocyte, one possibility is that Tdrd5l regulates *grk* in the nurse cells and in *tdrd5l* mutants, translation of Grk could be derepressed in the nurse cells. This might also cause less of the *grk* RNA to be localized to the developing oocyte, which could account for the decrease in oocyte staining I observed. The decrease in orb seen in *Tdrd5l* mutant oocytes could account for the variability I observed in the dorsal appendage phenotypes.

Another maternal RNA of interest is *osk*, which localizes to the posterior of the developing oocyte where it is translated during oogenesis (Ephrussi, Dickinson, and Lehmann 1991). Similar to the assay used to investigate regulation of *grk*, we immunostained control ovaries and *tdrd5l* trans-heterozygous mutant ovaries for Osk

protein. As expected, in control ovaries Osk protein localized to the posterior of late-stage egg chambers and was not detected in mid-stage egg chambers (Fig4.5 E, F). In *tdrd5l* mutants, Osk protein was still found at the posterior of late egg chambers, but it was also detected in the center of middle-stage egg chambers (Fig4.5G, H, and J). Additionally, proper localization of the *osk* RNA is dependent on proper *grk* localization since, prior to localizing to the dorsal anterior corner, *grk* localizes to the posterior to be translated and specify the posterior follicle cells which can be read in detail in the following review. (reviewed by Merkle et al. 2020). Thus, any alteration to Osk expression could be due indirectly to Tdrd5l acting on *grk,* or directly to its acting on *osk*.

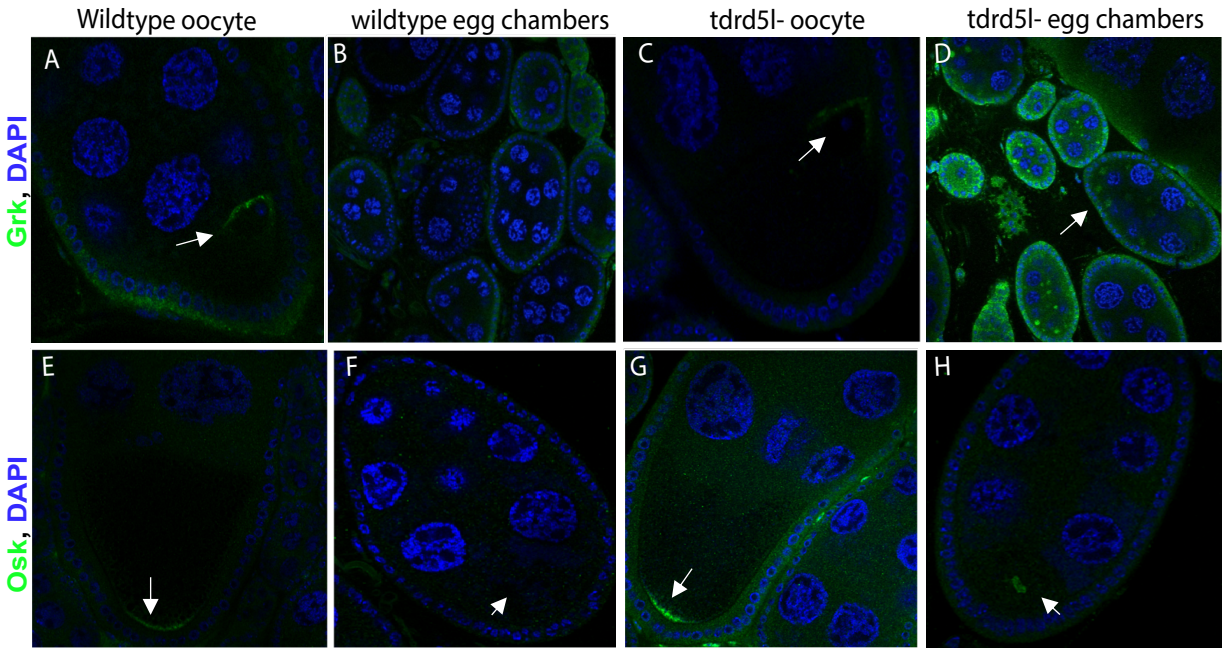

Wildtype oocyte
wildtype egg chambers
tdrd5l- oocyte
tdrd5l- egg chambers
Grk, DAPI
Osk, DAPI
A
B
C
D
E
F
G
H

Figure 4.5: Tdrd5l represses Grk and Osk protein expression

(A-H) immunofluorescence of proteins produced by maternally deposited RNAs using antibodies as indicated. A) Grk protein localizes to the dorsal anterior corner of wildtype oocytes. B) Grk protein is absent from wildtype nurse cells. C) diminished levels of Grk protein localize to the dorsal anterior corner of *tdrd5l*- oocytes. D) Grk protein was present in nurse cells of *tdrd5l*- egg chambers. E) Osk protein was localized to the posterior of wild type oocytes. F) Osk protein was absent from mid-stage wildtype nurse cells and oocytes. G) Osk protein is localized at increased levels to the posterior of *tdrd5l*- oocytes. H) Osk protein was localized to the center of mid-stage *tdrd5l*- oocytes.

The changes in Grk and Osk protein localization seen in *tdrd5l* mutant ovaries could be due to several possible mechanisms in which Tdrd5l might function. One potential explanation would be that *grk* and *osk* RNAs aren't trafficked to the oocyte as efficiently in *tdrd5l* mutants. Since we still get some protein in mutant oocytes, I know that transport isn't totally abolished. Another possibility could be that Tdrd5l usually represses translation in the nurse cells, so in the absence of Tdrd5l, *grk* can be translated in the nurse cells. To address the possibility of there being an RNA localization defect in *tdrd5l* mutants, I conducted fluorescence in sit hybridization (FISH) to visualize the *grk* and *osk* mRNAs in *tdrd5l* mutant ovaries.

FISH for the *grk* RNA in *tdrd5l* mutants revealed no changes in RNA localization or expression levels compared to wild type flies. (Fig 4.6A, and B; N= 15 ovaries per genptype). In wildtype ovaries I see enrichment of the *grk* RNA at the dorsal anterior corner of the developing oocyte. We see this same FISH signal in the *tdrd5l* mutant ovary oocytes. This observation suggests that mis-localization of *grk* RNA is not the cause of the Grk protein expression phenotype we see in *tdrd5l* mutants. One interesting possibility is that Tdrd5l could be regulating a regulator of the *grk* mRNA instead of directly acting on the *grk* mRNA itself. Contrary to what we observed when looking at *grk* mRNA localization, when we conducted FISH for the *osk* mRNA in *tdrd5l* mutants, we did see a change in mRNA localization. In 10% of *tdrd5l* mutants, the *osk* mRNA is not tightly localized to the posterior of the oocyte as it is in wildtype flies (Fig 4.6 C and D; N= 12 gonads per genotype). Based on this finding, I propose two possible explanations. First, Tdrd5l could be regulating *osk* mRNA independently of its regulation

of *grk*; this scenario would mean that Tdrd5l is acting in two separate pathways. Another possibility is that in *tdrd5l* mutants, Grk doesn't specify the posterior of the egg chamber, and this failure leads to defects in microtubule organization that result in *osk* mRNA and protein localizing to the center of the oocyte {Merkle et al. 2020]. To further investigate this possibility, I conducted an assay to see if the posterior follicle cells are specified in *tdrd5l* mutant ovaries.

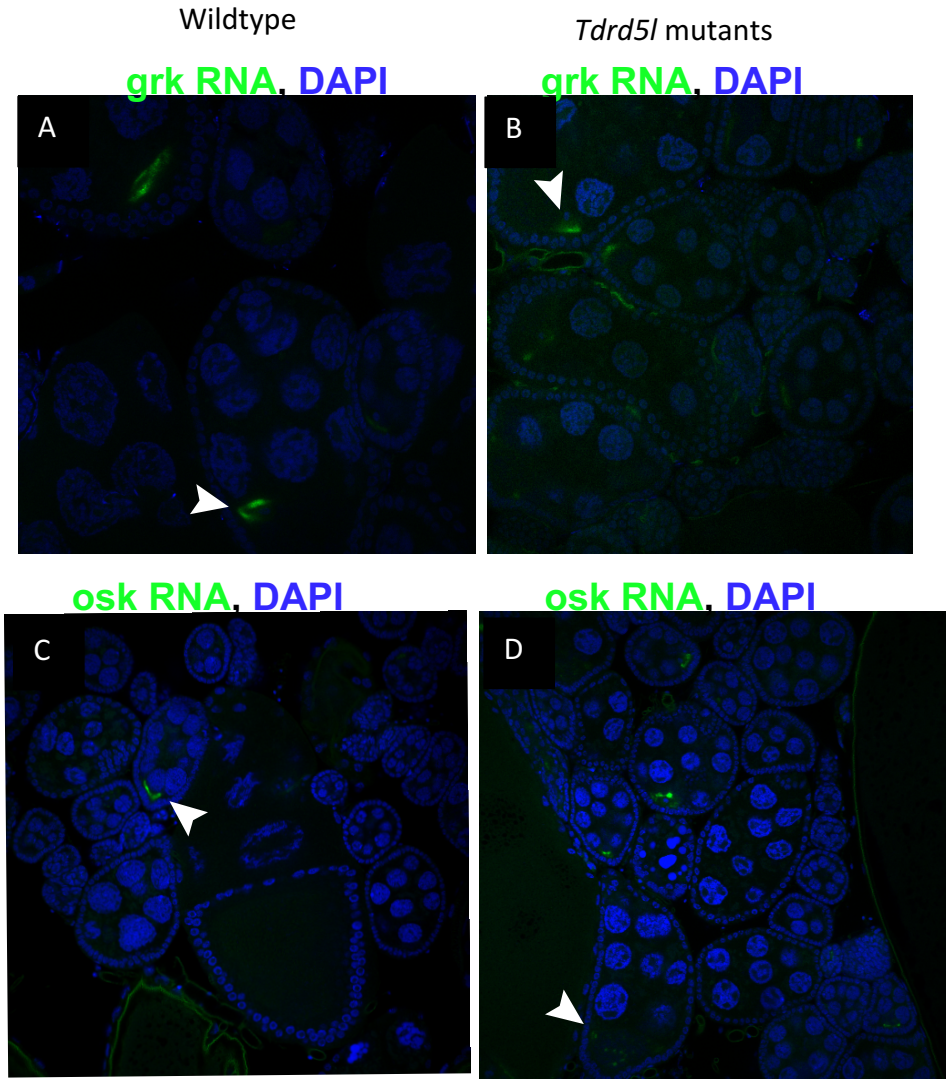


Figure 4.6 Tdrd5l does not alter *grk* RNA localization but does for *osk*

FISH for *grk* and *osk* RNAs, all RNA probes are shown in green. (A) *grk* RNA localizes to the dorsal anterior corner of the oocyte in wild type flies and in (B) mutant flies. (C) *osk* localizes to the posterior of the developing oocyte in wild type flies, but (D) can occasionally be seen dispersed throughout the oocyte in *tdrd5l* mutants

Tdrd5l represses protein expression of Orb, the *grk* activator

Ectopic expression of Grk in the nurse cells of *tdrd5l* mutant ovaries suggests that Tdrd5l directly binds to the *grk* mRNA to repress its translation. Since there is no RNA binding domain in Tdrd5l, it is more likely that Tdrd5l indirectly represses the *grk* mRNA by either repressing an activator of Grk translation or activating a repressor of Grk translation. The most well characterized activator of Grk translation is the cytoplasmic polyadenylation element bind protein (CPEB) Orb. Like *grk*, the *orb* mRNA is repressed in the nurse cells but is highly translated after deposition into the oocyte. Furthermore, Grk translation in the oocyte requires the presence of the Orb protein, and ectopic expression of Orb in the nurse cells is sufficient to trigger Grk translation in the nurse cells(Davidson et al. 2016).

To determine if mis-regulation of Grk in *tdrd5l* mutants might be due to Tdrd5l regulation of Orb, I immunostained control and *tdrd5l* trans-heterozygous mutant ovaries. As expected, control ovaries displayed high levels of Orb staining in oocytes with very low levels of Orb protein expression in the most posterior nurse cell (Fig4.7A). In *tdrd5l* mutant ovaries, Orb protein was detected in the oocyte but also was expressed in all the nurse cells of each egg chamber. Additionally, this ectopic Orb staining detected in mutants was not diffuse staining, but rather, large blobs of Orb in the nurse cell cytoplasm and less evenly diffuse staining in the oocyte (Fig 4.7B).

Since Orb protein is ectopically expressed in *tdrd5l* mutants, I tested to see if *tdrd5l* and *orb* genetically interact with each other. The first assay we conducted to answer this question was to compare *orb* RNAi in a wildtype background and in a *tdrd5l*

-/+ background. *orb* RNAi in a wildtype ovary results in the loss of egg chambers with only the early germline present (Fig 4.8A). However, when *orb RNAi* was conducted in the *tdrd5l-/+* ovaries, some of the early and mid egg chambers were rescued, compared to *orb* RNAi where only germaria are present (Fig 4.8B-C). Additionally, all ovarioles were rescued in a rescued ovary. This suggests that *tdrd5l* and *orb* genetically interact in such a way that loss of Tdrd5l function is able to partially rescue loss of Orb function in the ovary.

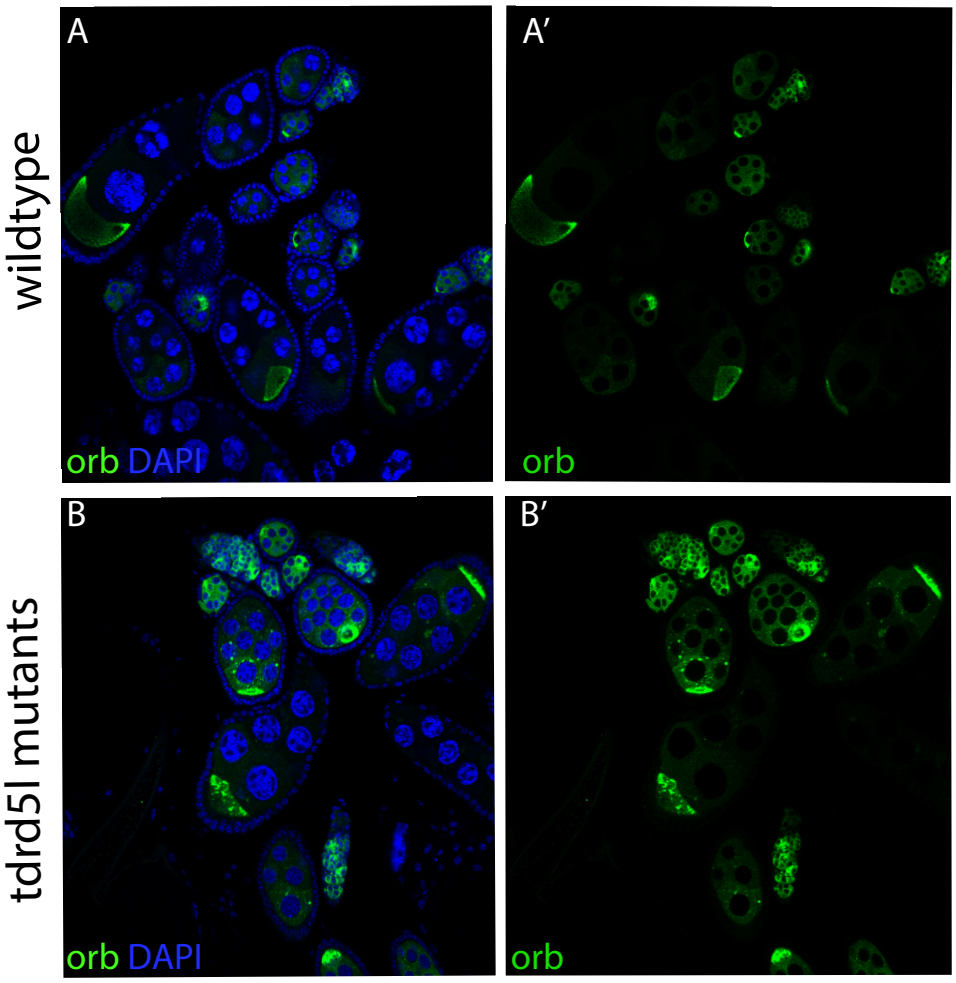


Figure 4.7: Tdrd5l represses Orb protein expression in nurse cells

(A) Orb staining is mostly restricted to the oocyte in later egg chambers of wildtype ovaries. (B) Orb protein staining is present in oocyte and nurse cells of *tdrd5l* mutant ovaries.

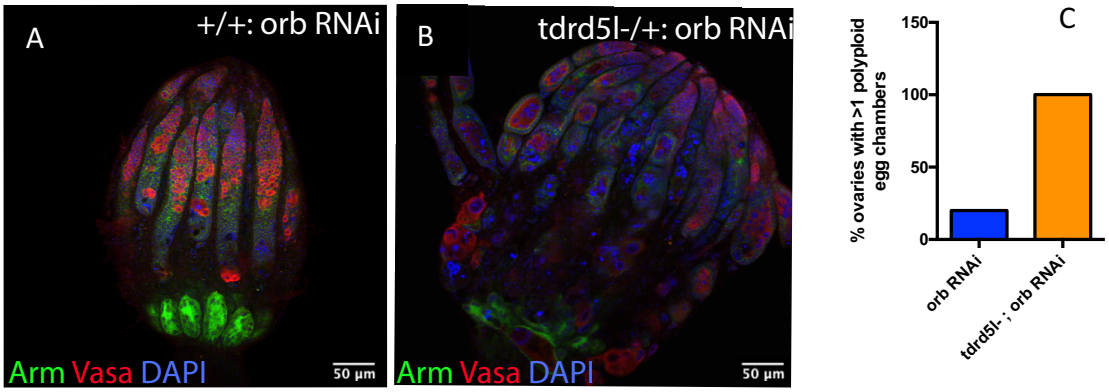


Figure 4.8: The *orb* RNAi phenotype is partially rescued in *tdrd5l* mutants

(A) *orb* RNAi results in a germline-loss phenotype with almost no polyploid egg chambers as quantified in (C). (B) *orb* RNAi conducted in a *tdrd5l* mutant background has a less severe phenotype as quantified in (C). N = 20 gonads per genotype.

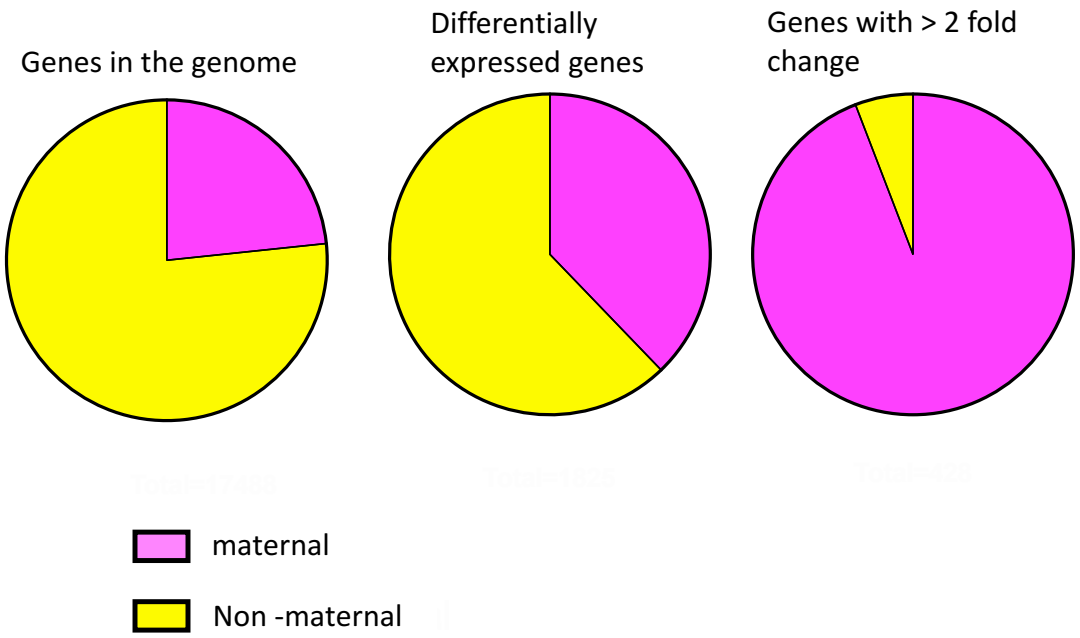
Tdrd5l regulates maternally deposited transcripts

To determine the extent to which maternally deposited RNAs were represented in the population of significantly changed genes, I overlapped the list of significant genes with a list of genes present in the 0-2hr old *Drosophila* embryo since this time point is before the onset of zygotic transcription. We compared our differentially expressed genes in three ways. First, I compared all differentially expressed (significance described in methods) genes to maternally deposited RNAs and found that ~ 30% of differentially expressed genes in *tdrd5l* mutants are maternally deposited (Fig4.9A), this was 690 out of 1825 genes that were increased or decreased in expression. Next, we looked at our most highly differentially expressed genes and compared those to the list of maternally deposited RNAs. Of the differentially expressed genes with a log2-fold change of 2 or greater, roughly 94% (171 of 182) of those genes were maternally deposited RNAs (Fig4.9A). Most differentially expressed genes with a log2-fold change of 1 or greater roughly 95% (403 of 428) were also maternally deposited (Fig 4.9A).

A common feature of almost all maternally deposited RNAs is that they are transcribed in the nurse cells and then are transported to the oocyte. This transport mechanism relies on adapter proteins that bind to the RNAs and hook them onto transport proteins; these transport proteins then walk the RNAs along the cytoskeleton and through the ring canals into the oocyte. One of the major adaptor/transport complexes is the BicD/Egl complex. Recently (Vazquez-Pianzola et al. 2017) employed a RNA immunoprecipitation followed by sequencing (RIPseq) approach to determine which mRNAs were pulled down by this complex. Since many RNAs bound to this

complex are hypothesized to be maternally deposited, we overlapped the top 100 hits from their RIP experiment with our *tdrd5l* mutant RNAseq. We found that ~30% of their top 100 RNAs were also differentially expressed in *Tdrd5l* mutant ovaries (Fig 4.9B). Taking these analyses together, our data suggest there is a significant de-repression of maternally deposited RNAs in *tdrd5l* mutants. The small change in expression level with these RNAs is consistent with the fact that most of these RNAs are post-transcriptionally regulated, thus if that is the level of regulation that Tdrd5l is acting on, there wouldn't be any large changes is transcription.

A


Genes in the genome
Differentially expressed genes
Genes with > 2 fold change
maternal
Non -maternal

B

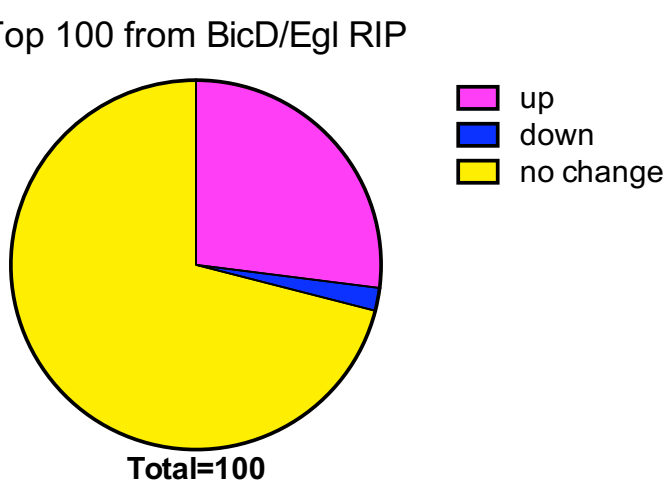

Top 100 from BicD/Egl RIP
up
down
no change
Total=100

Figure 4.2: overlap of maternally deposited RNA lists with *tdrd5l* mutant RNAseq

A) the number of differentially-expressed genes that are maternal are marked in magenta, and non-maternal are marked in yellow. The first pie chart represents all genes in the genome. (B) Shows the overlap between genes considered to be differentially expressed in *tdrd5l* mutants compared to a published BicD/Egl RIP experiment (Vazquez-Pianzola et al. 2017).

Discussion

The work detailed in this chapter describes new functions of Tdrd5l, a relatively new gene identified in our lab. Previous work had demonstrated the ability of Tdrd5l to promote male identity in the XY germline(Primus et al. 2019). My work here furthers that initial study and demonstrated that Tdrd5l has additional functions in the female germline separate from its role in promoting male fate. This role is different from other genes in germline sex determination, such as Sxl and Phf7, which only function in either the female or male germline respectively(Yang, Baxter, and Van Doren 2012, 7).

When I assayed for phenotypes in the post meiotic germline based on when post-transcriptional gene regulation is most important in males and females, we found phenotypes in *tdrd5l* mutants of both sexes.

The males exhibit disrupted spermatid elongation, a problem that could indicate faulty spermiogenesis. This phenotype warrants further study, both to understand exactly what step of spermatid elongation is disputed, and to potentially identify target RNAs of the Tdrd5l RNP in the male germline.

Close examination of eggs laid by *tdrd5l* mutants revealed improper development of the dorsal appendages, suggesting that the dorsal/ventral axis was not properly specified in the developing *tdrd5l* oocytes. These data suggested that *gurken* RNA is mis-regulated in. This result also brings into question whether all the body axes

are specified correctly in eggs laid by *tdrd5l* mutants, and it suggests an interesting avenue of future work on *tdrd5l*.

Investigation into the role of Tdrd5l in regulating specific maternally deposited RNAs revealed that Tdrd5l regulates both *grk* and *osk* at the protein level. We observed ectopic expression of the Grk protein in nurse cells and mis-localized expression of the Osk protein in *tdrd5l* mutant oocytes. These observations led me to question whether regulation of these RNAs was due to Tdrd5l altering their localization. FISH showed that while there was no change in *grk* RNA localization in *tdrd5l* mutants, *osk* RNA was mis-localized in the oocytes of *tdrd5l* mutants. This result is particularly interesting since Tdrd5l regulation of *osk* could be independent of Tdrd5l regulation of *grk*, or it could be a consequence of it. Since Grk protein is first translated at the posterior of the oocyte to specify the posterior follicle cells before it specifies the D/V axis, one possibility is that in *tdrd5l* mutants the posterior isn't properly specified; thus, *osk* is mis -localized to the center of the oocyte.

To further understand the mechanism that Tdrd5l uses to regulate *grk*, I took a step up the pathway of *grk* regulation to determine if this regulation by Tdrd5l is indirect. I identified increased expression of Orb protein in the nurse cells of *tdrd5l* mutant ovaries, suggesting that Tdrd5l regulates the *grk* RNA indirectly through regulation of its activator Orb. Experiments to address this regulation of *grk* are currently focused on Cup and dFMR1, which both act to silence Orb in the nurse cells.

The data shown here are a few specific examples of Tdrd5l regulation of maternally deposited RNAs. These results raise a number if interesting avenues for

further investigation such as whether axis specification is affected in all directions. Data that I described here demonstrated that there is a hatching defect in embryos from *tdrd5l* mutant mothers and that some of these eggs have dorsal appendage defects that could be due to mis-regulation of *grk.* This latter phenotype raises the possibility that the hatching defect could be due to improper axis specification.

Another outstanding question to consider when taking the maternally deposited RNA results together with Tdrd5l localization is whether these maternally deposited RNAs such as *grk, osk,* and *orb* physically localize to the Tdrd5l granules. If they do overlap, then that provides evidence that regulation by Tdrd5l is direct. If they do not overlap, then the other RNAs that occupy these granules might provide some mechanistic insight since those genes could lie between Tdrd5l and RNAs such as *grk* in a regulatory cascade. since data from chapter 3 shows that alleles of Tdrd5l that do not localize to a granule also behave as mutant alleles functionally, then whatever Tdrd5l does in its granule is required for its function.

Chapter 5: Conclusions and Discussion

The goal of this study was to advance our understanding of a recently identified male-promoting germline gene, *Tdrd5l*. A major advancement with this work was uncovering an unexpected role for Tdrd5l in female germline development.

The initial RNAseq screen that identified Tdrd5l was conducted in cells that highly express Sxl, which is a known repressor of *Tdrd5l(Primus et al. 2019).* The immunohistochemistry analysis of Tdrd5l conducted in this study revealed that Tdrd5l is indeed not expressed in the female germline cells used in the RNAseq screen, but rather, it is expressed in cystoblasts that are one cell division later in germline development. This observation explains why we initially thought *tdrd5l* might be a male-specific gene. Due to these results, I suggest that Tdrd5l is responsible for promoting male identity in germline stem cells (GSCs) while having a more general role in germline development later. This idea is yet to be experimentally tested and will be an interesting avenue of future work.

Tdrd5l localizes to a novel RNA granule

Using a variety of tagged alleles and an antibody against Tdrd5l, I observed that Tdrd5l localized to granule structures in the germline of both males and females. These granules appeared perfectly spherical, similar to published RNA granules that phase transition to form membrane-less organelles(Molliex et al. 2015). I demonstrated that, while Tdrd5l granules are present in both the male and female germline, the granules first appear in the GSCs in males and in the cystoblasts in females.

The size of the Tdrd5l granule is roughly 1 micron in diameter, a size that is much larger than many known RNA granules aside from stress granules (Chernov et al. 2009).

Additionally, Tdrd5l only localizes to the periphery of this granule and is absent from the center. Such staining has only been observed for two other granules. First, SMN occupies the outer layer of the U body in the female germline (J.-L. Liu and Gall 2007), similar to the way Tdrd5l occupies its granule. Second, certain proteins such as Vasa and Aubergine (Aub), which reside in a nuage-like granule in the male germline called the piNG body, also localize this way(Kibanov et al. 2011). One difference between the Tdrd5l granule and the pING body is that other proteins are seen to occupy the center of the piNG body, but none of our experiments uncovered a protein that localizes to the interior of the Tdrd5l granule (so far).

In addition to these larger granules where Tdrd5l only localizes to the periphery, Tdrd5l also occupies smaller granules with no obvious substructure. These small granules account for most of the granules observed in the female germline. In the male germline I observed a transition in granule types from small to large granules. The small granules were present predominately in the early male germline together with a few of the larger ones, however by the time development reached the 16-cell cyst, there were far fewer small granules and mostly larger granules.

I hypothesize several possible mechanisms to explain these differences in size and shape hinted at by my data. First, the small and large granules could represent two different populations of Tdrd5l granules, each with different functions. Data presented in this dissertation that support this idea include the localization of SMN to the smaller granules but not the larger ones. Additionally, Dcp1 is associated only with the larger granules. A second possibility is that the smaller granules represent an immature

population that grows in size with development. We have no evidence yet in support of this second idea, but it would be an interesting idea to further investigate, however this idea would not account for the differences in proteins we see localizing to the different Tdrd5l granule populations.

To determine if Tdrd5l granules represent a known type of RNA granule, I co stained gonads to analyze proteins that are hallmarks of the most well-characterized granules. Since many tudor-domain-containing proteins, including the closest homologues of Tdrd5l, localize to the nuage(Patil and Kai 2010), I co stained for Tdrd5l and Vasa. As shown in chapter 2, Tdrd5l does not appear to localize in the nuage, but appears to closely associate with Vasa staining nuage. The same pattern has been seen in the lab for Aub and Ago3, two proteins known to function in the piRNA pathway. Both Aub and Ago3 co-localize with the nuage but not with Tdrd5l.

Next, we asked whether Tdrd5l granules were a population of processing bodies. To answer this question, we co-stained with Dcp1, which localized with the periphery of Tdrd5l granules but not to the interior of the granules. It should also be noted that Tdrd5l granules are roughly 3 times larger in diameter than processing bodies; thus, it is highly unlikely that Tdrd5l granules are processing bodies.

Lastly, based on previously published data on SMN data(J.-L. Liu and Gall 2007), we asked if Tdrd5l co localizes with SMN in the U bodies. Interestingly, while most Tdrd5l granules did not co localize with SMN, a sub population of the smaller Tdrd5l granules overlapped with SMN granules. This line of evidence suggests that Tdrd5l could associate with distinct sub populations of granules.

Based on my data, which demonstrated no perfect co-localization between Tdrd5l granules and known RNA granule markers, I hypothesize that Tdrd5l occupies a previously uncharacterized granule.

This exciting prospect leaves me with many unanswered questions. I do not know what other factors occupy the granule(s). Based on my RNase A assay, which demonstrated Tdrd5l granules are not observed following treatment with RNase, I assume that there is RNA, but I do not know what RNAs or what proteins populate Tdrd5l granules. The next steps in fully understanding Tdrd5l granules will be to identify proteins that either complex with Tdrd5l or occupy its granule. Eventually I would want to conduct experiments to identify the RNAs that reside in this granule. It will be interesting to determine if there are specific RNAs that localize to the granule or if the granules contain a more general population of RNAs. Another important aspect of these studies will be to compare proteins and RNAs that occupy Tdrd5l granules in the male and female germline. Based on when I observe these granules during gametogenesis and on known RNAs that are regulated at those times, I would conduct targeted experiments that assess those specific candidate factors.

Tdrd5l regulates maternally deposited RNAs

The experiments highlighted in chapter 4 demonstrate that Tdrd5l plays an important role in regulating certain maternally deposited RNAs. While the RNAseq data comparing mutant to control ovaries suggested there could be a broader role for Tdrd5l

with respect to maternal RNA deposition, I was able to gain the most insight into its regulation of *grk.*

Tdrd5l regulation of *grk*

To understand Tdrd5l function in the development of the female germline, I conducted egg-lay assays to check for differences in the number of eggs laid by *Tdrd5l* mutants compared to wildtype flies. I also used these egg-lay assays to check for differences in hatch rate and for defects in egg/embryo morphology. My assays showed that not only are there fewer eggs laid by *Tdrd5l* mutants, but of those laid, far fewer hatched. To determine the cause of this hatching defect I looked for any obvious defects in the eggs that were laid.

Drosophila eggs have 2 eggshell structures called dorsal appendages; these appendages reside on the dorsal side of the egg and are used for respiration by the developing embryo. Synthesis of the appendages requires the activity of Grk in the oocyte, which signals to the overlying follicles to specify dorsal follicle-cell fate. If insufficient Grk is present in the oocyte, the eggs will form no appendage or a single appendage indicative of a ventralized egg (F Shira Neuman-Silberberg and Sch, 1993). If Grk isn't localized tightly enough to the dorsal anterior corner of the egg, a crown or ring-like appendage will form indicative of a dorsalized egg. In eggs laid by *Tdrd5l* mutants, many exhibited these dorsal appendage phenotypes; even more striking was that these eggs made up a significant portion of the unhatched eggs laid by *Tdrd5l* mutants. These results suggested that *grk* was mis-regulated in *Tdrd5l* mutants.

Like most maternally deposited RNAs, *grk* is transcribed in the nurse cells and kept translationally silenced during transport to the oocyte. There is no Grk protein translated in the nurse cells (Wong and Schedl 2011). Once in the oocyte, careful regulation of *grk* continues as it first localizes to the posterior and is translated and then secreted to specify the posterior follicle cells (Clouse, Ferguson, and Schüpbach 2008). By Stage 8 of oogenesis the *grk* mRNA has re-localized to the dorsal anterior corner of the oocyte where it is again is translated and secreted, this time to specify the dorsal side of the dorsal-ventral axis (Reviewed by Merkle et al. 2020).

In chapter 4 of this dissertation, I showed that in *tdrd5l* mutants Grk protein was ectopically expressed in nurse cells, suggesting that Tdrd5l acts to repress Grk protein expression either directly or indirectly. In the oocytes of *tdrd5l* mutants I saw no change in Grk protein localization, and FISH for the *grk* mRNA in *tdrd5l* mutant suggested there was no defect in mRNA localization. Based on previous work done by numerous labs that has uncovered the mechanism of *grk* regulation in the nurse cells and oocyte (Merkle review), I investigated if Tdrd5l regulates any of the factors that either silence or activate Grk protein expression.

The cytoplasmic polyadenylation element binding protein Orb is expressed highly in the oocyte and is repressed in nurse cells(Wong and Schedl 2011). Orb normally functions in the oocyte to bind the *grk* 3' UTR and recruit Wisp to lengthen the polyA tail and activate *grk* translation(Norvell et al. 2015). Thus, ectopic Orb expression in nurse cells is sufficient to activate Grk protein expression in nurse cells as well(Davidson et al. 2016). Since loss of *tdrd5l* function produced ectopic Grk in nurse cells similar to the

gain-of function phenotype produced by Orb, Orb was an attractive candidate for further exploration.

To test if Orb expression was altered in *Tdrd5l* mutants, I stained for Orb in adult ovaries and saw much higher expression of Orb protein in nurse cells compared to wildtype ovaries. While this result suggested that Tdrd5l might regulate *grk* through its regulation of *orb*, there are still other ways Tdrd5l can exert its regulation of *grk.* For example, Orb is kept translationally repressed in nurse cells by dFMR1 and Cup(Costa et al. 2005), and Tdrd5l could act by regulating these factors (although preliminary data are inconclusive as to whether dFMR1 expression is altered in Tdrd5l mutants). Additionally, the Squid is required for *grk* repression in nurse cells(Clouse, Ferguson, and Schüpbach 2008). While I am just beginning my investigation on Squid, this relationship provides another possible mechanism for how Tdrd5l might regulate *grk.*

Mis-regulation of *osk* in *Tdrd5l* mutants

Another maternally deposited RNA I investigated was *oskar(osk).* Osk protein is required to specify the germplasm and recruit in posterior factors(Ephrussi, Dickinson, and Lehmann 1991; J. L. Smith, Wilson, and Macdonald 1992). In wildtype flies *osk* mRNA is synthesized in the nurse cells and kept translationally silent until it is localized to the posterior of the developing oocyte(Nakamura, Sato, and Hanyu-Nakamura 2004). In *Tdrd5l* mutants, some Osk protein was distributed away from the posterior of the oocyte, and FISH for *osk* mRNA in *Tdrd5l* mutants also showed that some *osk* RNA did not localize as tightly to the posterior of the developing oocyte as seen in wildtype flies. While this regulation of *osk* by Tdrd5l could be independent of Tdrd5l regulation of *grk*,

it is possible that the mis-regulation of *grk* in *Tdrd5l* mutants is the cause of the mis-localization of *osk* mRNA and protein. Since Grk is responsible for specifying the posterior follicle cells and thus the posterior domain of the oocyte, if that specification does not occur, then the *osk* mRNA would not localize to the posterior of the oocyte and thus would not be translated there. While these hypotheses cannot be distinguished at this point, I have obtained reagents to do the necessary experiments to determine if the posterior follicle cells are specified in *Tdrd5l* mutants.

Final comments on maternal RNAs

While in this study I have only uncovered a role for Tdrd5l regulation of three maternally deposited RNAs (*orb, grk, osk)* either directly or indirectly, I do not know how specific the function of Tdrd5l is for these RNAs. Studies on Orb have also shown that it is required for the activation of Osk translation, thus one interesting possibility is that Tdrd5l regulates Orb which then in turn has an effect on the target RNAs of Orb. Our RNAseq data show that maternally deposited RNAs are overrepresented in the genes that are differentially expressed between *Tdrd5l* mutant and wildtype ovaries, and this result provides some support for a broader function of Tdrd5l in the regulation of maternally deposited RNAs.

Additionally, my genetic interaction screen to identify pathways in which Tdrd5l might function revealed that *Tdrd5l* genetically interacts with the deadenylase *twin*. Twin has a broad role in post-transcriptional gene regulation a method that is heavily used in the process of maternal RNA deposition. These mRNAs must be kept stable and silent until they reach the oocyte and often for long periods after their deposition.

Ample evidence demonstrates the need for Twin in regulation of these maternal mRNAs and for proper oogenesis in general (Chicoine et al. 2007; Morris et al. 2005), thus providing another piece of evidence in support of Tdrd5l having a role in regulating these mRNAs.

An outstanding question is how I link these RNAs back to the function of Tdrd5l in the granules it occupies. I have had difficulty visualizing Tdrd5l in the female germline due to its low expression levels, but further studies should be conducted to determine if Tdrd5l protein is localizing with these target mRNAs. This could be done through IF/FISH or by Co-IP If these mRNAs were to be detected in the Tdrd5l granules, it would suggest that these granules are used to protect and repress mRNAs during the process of maternal RNA deposition.

Relation of Tdrd5l to homologues

Tdrd5l is most closely related to mouse TDRD5 and the *Drosophila* TDRD5 homolog Tej. All three of these proteins contain an extended tudor domain, which is a common feature in germline expressed Tudor-domain-containing proteins. The major difference between Tdrd5l and its close relatives is that Tej and TDRD5 contain an N-terminal LOTUS domain, while Tdrd5l is not predicted to have such a domain. There are no predicted domains other than the C terminal tudor domain in Tdrd5l, however I suspect the N terminal half of the protein has some yet to be identified function. If I add any tag large or small to the N terminus of the protein, Tdrd5l behaves as a mutant allele and cannot localize to its granule without an untagged allele of Tdrd5l in the cell.

Additionally, secondary structure predictions of Tdrd5l predict there to be numerous alpha helices in this region, structures that also imply some functional significance.

In addition to domain architecture, our work shows that Tdrd5l does not function in the same pathway as Tej and diverges from mouse TDRD5 in some of its function as well. Both TDRD5 and Tej function in retrotransposon regulation(Patil and Kai 2010). The previous paper from our lab demonstrated through qRT-PCR of the most common transposons that Tdrd5l does not repress transposons in the male germline. I followed up on that finding using the RNAseq data produced in this dissertation to globally ask whether Tdrd5l regulates transposons in the male or female germline. In both cases there were no global changes in transposon expression levels in Tdrd5l mutants, however there were 4 transposons with changes in expression in *Tdrd5l* mutant females.

While Tej only regulates retro transposons in flies (Patil and Kai 2010), TDRD5 in mice regulates retrotransposons and coding mRNAs as well. These differences suggest that the dual functions of TDRD5 in mice could be split between Tej and Tdrd5l in flies. As mentioned in the introduction chapter, this sub-functionalization is thought to have happened with TDRD11. Similar to Tej, there is no known function for TDRD5 in female mice. Psi-BLAST of Tdrd5l also pulls up mouse TDRD1 as a potential relative, however there are three other fly tudor-domain-containing proteins that are predicted to be the TDRD1 homolog. Moreover, while Tdrd5l is most closely related to TDRD5 and its homologues, Tdrd5l clearly possesses unique functions compared to other Tdrd5 proteins.

Final comments on male function

While earlier work on Tdrd5l predicted it to be a male biased gene, this dissertation focused on the mechanism of Tdrd5l function in the female germline and only addressed localization of Tdrd5l in the male germline. There is still much work to be done to determine how Tdrd5l functions in the male germline, both in how it promotes male fate and if it has a function unrelated to male fate in later germline stages. Preliminary experiments have suggested that spermiogenesis phenotypes are present in *tdrd5l* mutant males. Since Tdrd5l expression stops prior to meiosis, one possibility is that Tdrd5l represses mRNAs needed after meiosis. Another piece of data in support of Tdrd5l repressing mRNAs needed for late spermatogenesis is the upregulation of Mst36Fb in *Tdrd5l* mutants as indicated by our RNAseq data. While much remains unanswered about the function of Tdrd5l in males, future experiments could show a role for Tdrd5l regulation of a special set of mRNAs required for later steps in gametogenesis, as we saw in the female germline.

Appendix 1: Genetic interaction results

To identify what pathways Tdrd5l might function in, I carried out a biased set of genetic interaction experiments as described in chapter 3. These genes included genes involved in posttranscriptional gene regulation, germline development, and meiotic arrest genes. The results of all the tested genes are included in the table below along with the corresponding Bloomington stock center stock number for each RNAi line. Enhanced refers to RNAi that resulted in a worsening phenotype in *Tdrd5l* mutants, suppression refers to lessening of the phenotype when RNAi was conducted in *Tdrd5l* mutants. I considered enhancements or suppression to be slight if less than 10% if the gonads showed a difference in phenotype between RNAi in a wildtype background compared to RNAi in a *Tdrd5l* mutant background.

Gene	**Interaction in males**	**Interaction in females**	**Bloomington stock number**
tral	none	none	38908
lost	none	none	55201
twin	enhance	Enhance	32490, 32901
Aly	none	None	36723
Cup	Slight rescue	None	35406
Orb	none	suppression	43143
Dcp2	none	None	34806
Lsm	none	None	55912
Orb2	none	None	56997
Taf12L	none	None	62863
Edc3	none	None	34853
Me31b	none	None	33675
Upf1	none	none	43144
Comr	none	None	42522
Exu	Slight rescue	None	41816

Nht	none	None	57448
Dcp1	none	Enhance	67874
Loki	Slight enhance	none	64442
Rump	none	None	42665
Sa	none	None	36579
Ago2	Slight enhance	None	34799
Bam	none	Slight suppression	33631
Bgcn	none	Enhance	36636
Cg11456	none	None	26240
Cu	none	none	61293
Dcr-1	rescue	None	34826
Dcr2	none	None	33656
Drosha	enhance	None	33657
Gawky	rescue	Enhance	34796
Gld2	none	None	57177
MeiP26	none	enhance	57268
Loqs	none	None	34781
Not3	none	None	34966
Not10	none	None	32957
Pan2	none	None	53249
Pasha	none	None	26293
Pum	none	none	36676
Smd1	none	None	34834
Smn	none	None	36621
Tej	none	None	36879
Vasa	none	None	32434, 30496, 34950
Wisp	None	none	43141
Art6	None	None	36831
Csul	none	Slight enhance	43200
Bcd	none	None	35478
bicC	none	Slight suppression	35631
Cg4666	none	None	55939
CG10911	none	None	64624
Pgc	none	None	33720
Rig	Slight enhance	Slight enhance	34684

snRNA: U2	none	none	64515

Table A1.1: Results for genetic interaction experiments

Genes tested for interactions with *Tdrd5l* are listed in the first column. The second column reports whether there was a genetic interaction in the male germline and in what way the genes interact. The third column reports the same information for female interactions. The last column contains the stock number used for the RNAi of interest

Appendix 2: RNA sequencing results

gene	sample_1	sample_2	value_1	value_2	log2(fold_change)
Mst36Fb	m4_mut	cas9_con	74.9538	0.149244	-8.97219
CG3397	m4_mut	cas9_con	10.1536	0.224705	-5.49782
CG43115	m4_mut	cas9_con	131.254	3.14139	-5.38481
CR45271	m4_mut	cas9_con	31.6237	1.21062	-4.70718
CG33258	m4_mut	cas9_con	6.54038	0.289589	-4.4973
CG8837	m4_mut	cas9_con	14.386	0.667413	-4.42994
Traf4	m4_mut	cas9_con	8.81066	0.530102	-4.05491
Hn	m4_mut	cas9_con	9.01883	0.590981	-3.93176
CR44545	m4_mut	cas9_con	15.8241	1.18045	-3.74471
CR44143	m4_mut	cas9_con	6.00333	0.521114	-3.52609
CR45587	m4_mut	cas9_con	34.1225	3.42031	-3.31852
CR44080	m4_mut	cas9_con	3.69287	0.426921	-3.1127
CR43298	m4_mut	cas9_con	25.1688	3.01611	-3.06087
ninaE	m4_mut	cas9_con	0.870322	0.114565	-2.92538
CR44343	m4_mut	cas9_con	5.87262	0.833939	-2.81599
scpr-B	m4_mut	cas9_con	463.533	74.6686	-2.6341
CR44076	m4_mut	cas9_con	9.12644	1.60184	-2.51032
CG8160	m4_mut	cas9_con	1.62904	0.289956	-2.49012
GluRIIB	m4_mut	cas9_con	0.588952	0.105753	-2.47745
CG31198	m4_mut	cas9_con	4.06497	0.767781	-2.40448
CR33221	m4_mut	cas9_con	8.81291	1.76095	-2.32326
CR45098	m4_mut	cas9_con	2.37231	0.507462	-2.22492
CR45389	m4_mut	cas9_con	36.1241	7.72818	-2.22476
CG3290	m4_mut	cas9_con	5.51819	1.18261	-2.22222
CG2694	m4_mut	cas9_con	8.71134	1.88513	-2.20823
CG2663	m4_mut	cas9_con	1.48795	0.335599	-2.14852
TyrR	m4_mut	cas9_con	1.07646	0.245529	-2.13232
antr	m4_mut	cas9_con	10.5696	2.5242	-2.06602
CG14443	m4_mut	cas9_con	12.3503	2.98302	-2.0497
Rdl	m4_mut	cas9_con	0.784862	0.191506	-2.03505
CR45236	m4_mut	cas9_con	43.0598	10.5398	-2.0305
CG30371	m4_mut	cas9_con	3.80316	0.934733	-2.02457
CG15067	m4_mut	cas9_con	5.08545	1.26082	-2.01201

Table A2.1: Genes that had a log 2-fold change of 2 or greater increase in expression in *Tdrd5l* mutant testes

gene	sample_1	sample_2	value_1	value_2	log2(fold_change)
CG8100	m4_mut	cas9_con	0.313472	30.6016	6.60913
CR45537	m4_mut	cas9_con	0.102392	4.90076	5.58083
CG13905	m4_mut	cas9_con	0.227998	9.24452	5.34151
CG33664	m4_mut	cas9_con	1.83335	69.9079	5.2529
CG34025	m4_mut	cas9_con	0.0891696	2.7849	4.96493
CG4020	m4_mut	cas9_con	0.117552	2.5567	4.44291
Osi14	m4_mut	cas9_con	0.0807378	1.3275	4.03933
CR44313	m4_mut	cas9_con	1.23217	15.9764	3.69667
CG15597	m4_mut	cas9_con	0.269394	3.07387	3.51227
Ir75a	m4_mut	cas9_con	0.0725096	0.75522	3.38065
CR44058	m4_mut	cas9_con	0.680047	6.36991	3.22756
CR44789	m4_mut	cas9_con	1.44319	11.8778	3.04093
CG12075	m4_mut	cas9_con	0.210969	1.65796	2.97431
SPR	m4_mut	cas9_con	0.358154	2.48771	2.79616
CR43652	m4_mut	cas9_con	1.1003	7.06641	2.68308
CG5945	m4_mut	cas9_con	0.272389	1.57821	2.53455
CR45706	m4_mut	cas9_con	0.202938	1.17499	2.53354
Ir75b	m4_mut	cas9_con	0.18918	1.06219	2.48921
CG6414	m4_mut	cas9_con	0.147197	0.814827	2.46874
CG12224	m4_mut	cas9_con	1.09453	5.9486	2.44224
Su(Ste):CR45795	m4_mut	cas9_con	0.500184	2.59565	2.37557
Meics	m4_mut	cas9_con	0.392056	1.95686	2.31941
CR43422	m4_mut	cas9_con	0.528127	2.48404	2.23373
28SrRNA-Psi:CR41609	m4_mut	cas9_con	106.5	496.505	2.22096
Appl	m4_mut	cas9_con	0.138207	0.625814	2.1789
PGRP-SB1	m4_mut	cas9_con	1.17573	5.30176	2.17291
CR44059	m4_mut	cas9_con	0.270668	1.19617	2.14383
Cpr76Bd	m4_mut	cas9_con	0.250203	1.0736	2.10129
CR45788	m4_mut	cas9_con	0.925304	3.91681	2.08168
28SrRNA-Psi:CR40741	m4_mut	cas9_con	598.776	2505.47	2.06499
CR43372	m4_mut	cas9_con	0.313408	1.297	2.04906
Gr93d	m4_mut	cas9_con	1.44671	5.82977	2.01066
CG14074	m4_mut	cas9_con	0.217093	0.873153	2.00792

Table A2.2: Genes that decreased in expression by log2-fold change of 2 or greater in *Tdrd5l* mutant testes

gene	sample_1	sample_2	Control	mutant	log2(fold_change)
CG4666	fc9	fm4	0.124037	10.6974	6.43035
CG13032	fc9	fm4	0.111466	9.00588	6.33619
CG16904	fc9	fm4	0.220936	15.3403	6.11756
Smvt	fc9	fm4	0.0721858	2.34588	5.02227
CG4757	fc9	fm4	0.221735	7.11793	5.00455
CG17562	fc9	fm4	0.268401	8.42276	4.97183
Dpt	fc9	fm4	0.567259	11.7881	4.37718
TotM	fc9	fm4	0.613371	12.2747	4.32278
v(2)k05816	fc9	fm4	0.217452	4.17552	4.26318
Vm34Ca	fc9	fm4	0.957171	17.5747	4.19858
CG17560	fc9	fm4	0.357254	6.55141	4.19678
CG16727	fc9	fm4	0.0614303	1.10567	4.16983
Amy-p	fc9	fm4	0.0692211	0.985442	3.83149
Osi15	fc9	fm4	0.706513	9.98003	3.82026
hkb	fc9	fm4	0.114274	1.53916	3.75158
CG42235	fc9	fm4	0.285638	3.71827	3.70237
Mur18B	fc9	fm4	1.31774	17.1233	3.69982
CG14292	fc9	fm4	1.69173	20.664	3.61055
CG7203	fc9	fm4	3.44295	38.5167	3.48377
AttB	fc9	fm4	1.82589	20.4253	3.48369
DptB	fc9	fm4	1.55046	17.3375	3.48312
Cpr100A	fc9	fm4	0.602066	6.49437	3.4312
Cpr92F	fc9	fm4	0.273096	2.93736	3.42704
CG11892	fc9	fm4	0.110556	1.14091	3.36734
CG2254	fc9	fm4	2.4967	24.7677	3.31037
CG6409	fc9	fm4	3.28995	32.6276	3.30996
CG8534	fc9	fm4	4.44767	43.1877	3.2795
Oatp58Dc	fc9	fm4	0.138901	1.34162	3.27185
Aph-4	fc9	fm4	1.66279	15.8693	3.25456
sosie	fc9	fm4	2.34949	21.2988	3.18035
CG13046	fc9	fm4	0.612534	5.38676	3.13656
CG7900	fc9	fm4	0.653258	5.69559	3.12412
CG42541	fc9	fm4	0.130928	1.10535	3.07765
Vm26Ab	fc9	fm4	10.1123	83.5344	3.04626
GstE14	fc9	fm4	4.87569	40.0917	3.03962
AttA	fc9	fm4	0.522878	4.13689	2.984
CG7910	fc9	fm4	1.62521	12.3865	2.93007
CG10514	fc9	fm4	0.252742	1.75986	2.79972
CG14567	fc9	fm4	0.656497	4.45808	2.76356

CG8147	fc9	fm4	0.293268	1.98166	2.75642
Dro	fc9	fm4	7.74464	51.8586	2.74331
CG17374	fc9	fm4	1.72631	11.4745	2.73267
CG34227	fc9	fm4	2.96255	19.3537	2.7077
AttC	fc9	fm4	1.72174	10.7442	2.64161
nAChRalpha4	fc9	fm4	1.07676	6.63319	2.62301
Yp2	fc9	fm4	57.6601	352.682	2.61272
CG7296	fc9	fm4	2.96552	16.6806	2.49181
CrzR	fc9	fm4	0.227161	1.27529	2.48903
CR45601	fc9	fm4	39.1109	214.104	2.45267
CG10560	fc9	fm4	0.304786	1.66238	2.44738
Yp1	fc9	fm4	11.1817	60.2027	2.42869
CG10553	fc9	fm4	0.284466	1.44633	2.34606
CG8736	fc9	fm4	1.74471	8.86577	2.34526
CG15531	fc9	fm4	1.87001	9.4221	2.333
CG5791	fc9	fm4	4.27918	20.942	2.29099
CG7084	fc9	fm4	0.550816	2.60502	2.24165
IM23	fc9	fm4	3.13875	14.5604	2.21379
cln3	fc9	fm4	0.382764	1.71688	2.16526
Vml	fc9	fm4	0.22861	0.976631	2.09493
CG10062	fc9	fm4	2.20311	9.17713	2.0585
CG5326	fc9	fm4	4.8229	19.6277	2.02491
CG1544	fc9	fm4	0.819553	3.2894	2.00492

Table A2.3: Genes with a log2-fold change of 2 or greater increase in *Tdrd5l* mutant ovaries

gene	sample_1	sample_2	Control	mutant	log2(fold_change)
CG10911	fc9	fm4	146.349	0.128135	-10.1575
CG43074	fc9	fm4	226.407	1.11658	-7.66369
Mur29B	fc9	fm4	7.52298	0.0679717	-6.79023
CG18088	fc9	fm4	7.45629	0.0692822	-6.74983
CG11878	fc9	fm4	17.2404	0.203818	-6.40237
CG30371	fc9	fm4	26.5007	0.331668	-6.32014
mesh	fc9	fm4	11.1615	0.142462	-6.29181
CG13321	fc9	fm4	37.1668	0.544197	-6.09374
GstD2	fc9	fm4	44.1353	0.839093	-5.71696
Damm	fc9	fm4	10.2797	0.227329	-5.49887
Pebp1	fc9	fm4	27.8002	0.73267	-5.24579
Oatp33Ea	fc9	fm4	3.42599	0.098041	-5.12699
CG12766	fc9	fm4	12.8413	0.390441	-5.03954
CG8907	fc9	fm4	2.1586	0.0720363	-4.90523
CG14989	fc9	fm4	1.54288	0.0564788	-4.77177
Cpr62Bb	fc9	fm4	5.68401	0.231989	-4.61478
CG7214	fc9	fm4	5.26165	0.224187	-4.55274
CG4301	fc9	fm4	1.0803	0.0524618	-4.36402
CG33926	fc9	fm4	6.44946	0.323085	-4.31919
CG12974	fc9	fm4	3.8779	0.214587	-4.17564
CG32700	fc9	fm4	1.2252	0.0724397	-4.08009
CG11893	fc9	fm4	13.0777	0.81748	-3.99978
CG44250	fc9	fm4	17.1547	1.08478	-3.98313
TyrR	fc9	fm4	0.65024	0.0418975	-3.95604
CG2681	fc9	fm4	1.52234	0.10872	-3.80761
CG6484	fc9	fm4	2.4177	0.185255	-3.70605
CG34198	fc9	fm4	4.20163	0.3229	-3.70179
CG3332	fc9	fm4	0.551712	0.0451913	-3.6098
CG13659	fc9	fm4	4.58455	0.389108	-3.55854
Cyp9b2	fc9	fm4	6.14336	0.521654	-3.55786
RpL22-like	fc9	fm4	2.38472	0.208954	-3.51256

primo-1	fc9	fm4	86.717	8.05863	-3.42771
GstD10	fc9	fm4	4.09631	0.422807	-3.27626
GstD5	fc9	fm4	5.04774	0.525144	-3.26485
CG34375	fc9	fm4	0.770773	0.0806617	-3.25635
CG9701	fc9	fm4	1.17778	0.124929	-3.23689
Kah	fc9	fm4	1.34109	0.144308	-3.21618
CG15152	fc9	fm4	5.64001	0.633047	-3.15531
CG5621	fc9	fm4	1.18847	0.133413	-3.15514
CG15784	fc9	fm4	127.702	14.6455	-3.12425
CG5945	fc9	fm4	7.38688	0.864625	-3.09482
neo	fc9	fm4	0.873636	0.104234	-3.06721
CG30401	fc9	fm4	1.09255	0.134749	-3.01935
Cyp309a1	fc9	fm4	1.39893	0.193176	-2.85634
CG31288	fc9	fm4	4.93973	0.687558	-2.84488
CG8100	fc9	fm4	1.39875	0.197878	-2.82145
AstC-R2	fc9	fm4	0.507045	0.0731542	-2.7931
CG5399	fc9	fm4	47.2338	7.21478	-2.71079
SkpC	fc9	fm4	7.81428	1.23759	-2.65858
CG18417	fc9	fm4	1.55779	0.276069	-2.4964
CG9498	fc9	fm4	13.6418	2.55734	-2.41531
CG8483	fc9	fm4	9.38023	1.76339	-2.41127
snRNA:U2:34ABa	fc9	fm4	128.464	24.5228	-2.38917
hbs	fc9	fm4	0.536855	0.104304	-2.36374
Cyp309a2	fc9	fm4	2.15376	0.419935	-2.35862
CG11619	fc9	fm4	1.02163	0.203649	-2.32672
Whamy	fc9	fm4	3.00675	0.621287	-2.27487
CG12782	fc9	fm4	2.65392	0.548766	-2.27386
CG9380	fc9	fm4	6.05278	1.26298	-2.26077
alphagamma-element:CR32865	fc9	fm4	8.87327	1.87856	-2.23984
pirk	fc9	fm4	7.22498	1.53887	-2.23112
CG32368	fc9	fm4	28.246	6.36535	-2.14974
CG5819	fc9	fm4	1.20725	0.28621	-2.07658
Eip78C	fc9	fm4	1.14777	0.274071	-2.06621
Hsp70Bc	fc9	fm4	6.74859	1.64686	-2.03487

CG8620	fc9	fm4	5.87108	1.45736	-2.01027
ple	fc9	fm4	3.74819	0.930635	-2.0099
CG9717	fc9	fm4	2.37863	0.593447	-2.00294
CG44014	fc9	fm4	9.95946	2.48863	-2.00071

Table A2.4: Genes with a log2-fold change of 2 or greater decease in expression in *Tdrd5l* mutants

Appendix 3: sexually dimorphic LncRNAs

Introduction

Long noncoding RNAs (LncRNAs) are a fairly new type of broad class of RNAs. A few of these RNAs such as Xist and H19 have been studied for over 50 years, but these RNAs were thought to be anomalies until the advent of next generation sequencing (NGS) technology. With NGS, LncRNAs were identified as a large class of non-coding RNAs longer than 200nt in length. Many of these RNAs came from what used to be thought of as "Junk DNA". To date numerous studies (C. Lin et al. 2017; Long et al. 2017; Dykes and Emanueli 2017)have found functions for lncRNAs in transcriptional and post transcriptional regulation. Some specific processes they work in include, cancer and development, often by changing the epigenetic landscape or by acting as miRNA sponges to buffer miRNA levels in the cell (Xu and Zhang 2017; Joo et al. 2019).

In *Drosophila* the two most well-studied LncRNAs are Rox1 and Rox2, both of which play a role in hyper-transcribing the X chromosome in males to bring the expression level of X genes to the same level as in females, which have 2 X chromosomes. To understand differences in gene expression between male and females in the germline, our lab conducted an RNAseq screen to identify male and female biased genes. This screen identified many protein coding genes of interest, but it also discovered many genes annotated as noncoding RNAs.

Methods

RNAseq reads from *bam* mutant males and females were mapped to version 6 of the *Drosophila melanogaster* genome using tophat (D. Kim et al. 2013). Differential gene expression analysis was conducted using cuffdiff (Trapnell et al. 2012). Mimic insertion fly lines were used for phenotypic analysis of selected LncRNAs. Dissected gonads were fixed and stained with mouse anti Arm, rabbit anti Vasa, Guinea pig anti Traffic jam, and DAPI. RT-PCRs were conducted using RNA bee extraction and superscript II for reverse transcription [Source]. CRISPR/Cas9 was used to create flies with a deletion for CR43684 using reagents from the fly CRISPR community (Gratz et al. 2015).

Results

To identify lncRNA genes in the RNAseq data set, the RNAseq reads were re-mapped to the newest version of the *Drosophila* genome. A differential expression analysis was done to identify male and female biased genes including lncRNAs. Our analysis uncovered ~140 LncRNA genes as either male or female biased. To confirm the RNAseq results, RT-PCR was conducted to validate sex biased expression of lncRNAs of interest. (Fig A3.1).

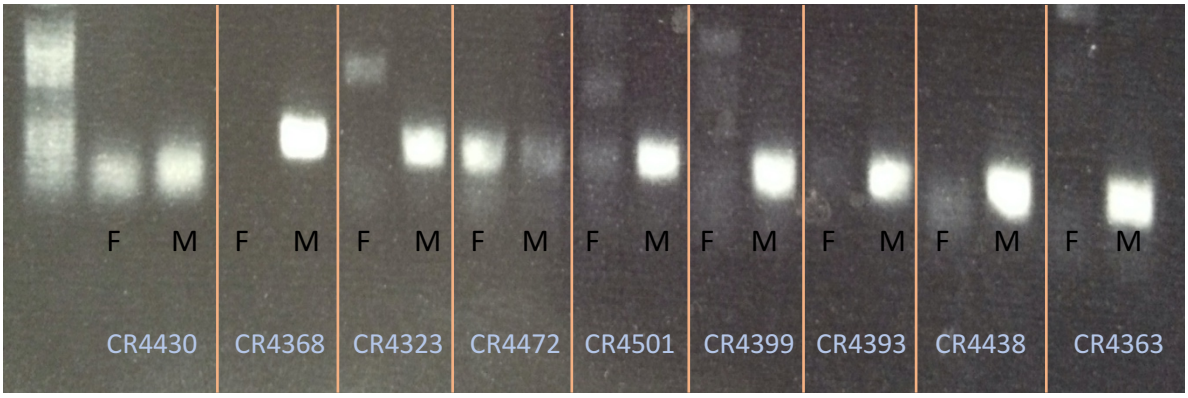


Figure A3.1 RT PCR of sex biased link RNAs

To determine if these lnRNAs have sex-specific gonad phenotypes, I used Mimic insertion flies that were predicted to have a Minos insertion in the 5' end of the gene or in the promoter. The rationale for using Mimic flies instead of RNAi for germline specific knockdown was that the cellular location of the selected lncRNAs was unknown, and most LncRNAs localize to the nucleus while the RNAi machinery localizes to the cytoplasm. The RNAs we chose to study with this method were CR43626, CR43334, CR44030, CR44366, CR43301, CR44347, CR32194, CR43593, CR44560, CR43299, CR45323, CR43239, CR45013, CR45013, CR44724, CR43932, and CR44051. Using the Mimic line method, I was also limited to what RNAs I could investigate, thus some LncRNAs on my list were not studied due to reagent availability.

To detect sex-biased phenotypes in the Mimic flies, flies were aged for 5-10 days at 25^{o}C before their gonads were dissected. Gonads were dissected and fixed according to methods used in (Gonczy, Matunis, and DiNardo 1997). Fixed gonads were then stained for Vasa, Arm, TJ, and DAPI to identify morphological defects. In addition to imaging for morphological defects, I also assayed for fertility. Only one line, the Mimic line for CR43299, was sterile.

Using our Mimic lines, I found a few LncRNAs that had morphological defects in the germline. The first one I assayed, CR45323, was homozygous for the Mimic insertion and had germline loss in males and egg-chamber tumors in females (Figure A3.2) I then crossed this Mimic line to a deficiency to test if the phenotype I observed was due to loss of function of the lncRNA of interest. Flies that were trans-heterozygous for the

deficiency and mimic line had a wildtype phenotype (Figure A3.3). Thus, the phenotype exhibited by Mimic homozygotes was not due to loss of the CR45323 transcript.

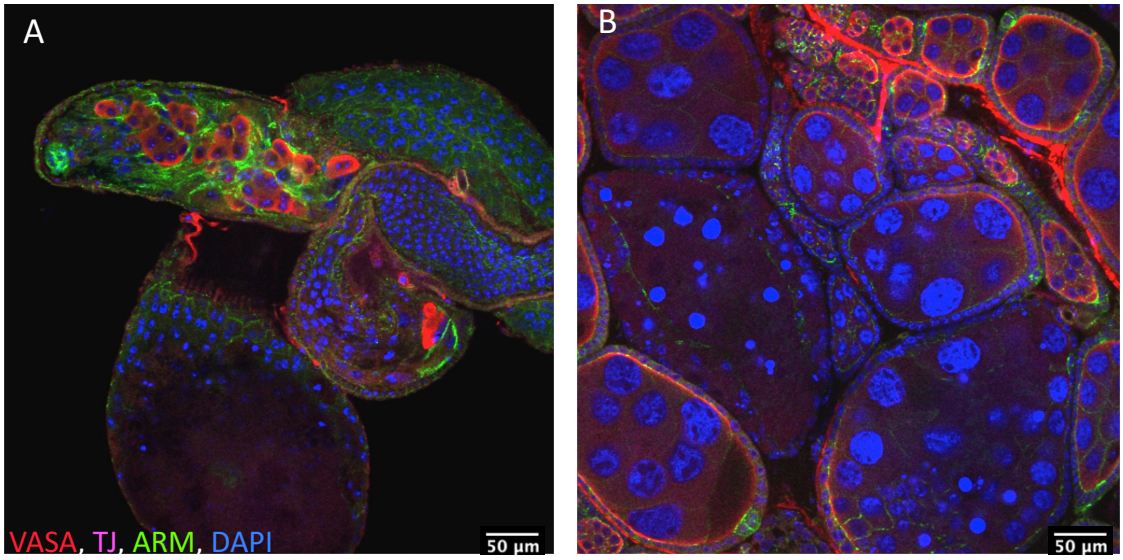


Figure A3.2: Mimic insertion in the *CR45323* locus results in gonad morphology phenotypes

(A) testes from flies homozygous for CR45323 mimic insertion have a germline loss phenotype. (B) Homozygous females displayed egg chambers with too many nuclei and cell death.

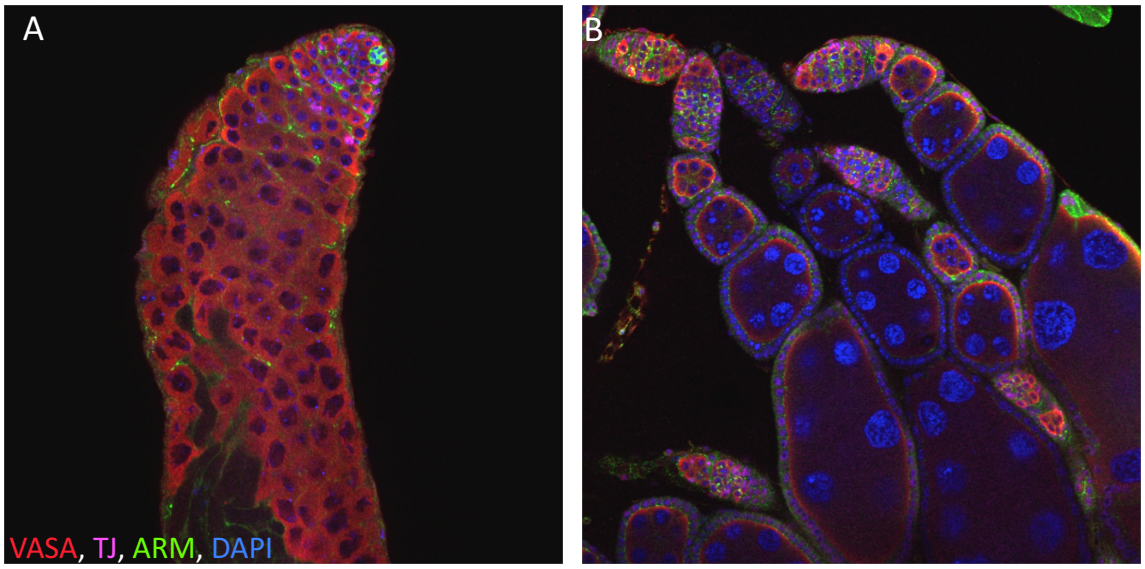


Figure A3.3 Mimic insertion in *CR45323* over a deficiency rescues the mimic phenotype

Expressing the mimic insertion in CR45323 over a deficiency for the lncRNA rescued the phenotype in (A) males and (B) females.

One LncRNA in particular that was highly enriched in the male germline but had no reagents available was CR43684. To study the potential function of this gene I planned to create a deletion allele using the CRISPR/Cas9 method. Due to time constraints, cloning of the CRISPR repair construct was never completed.

Discussion

While there are numerous lncRNAs that appear to be expressed in a sex-specific manner, my study of these RNAs was technologically limited. RNAi is usually not effective against lncRNAs since many are localized to the nucleus. My use of Mimic lines to disrupt the function of some of these lncRNAs was unsuccessful since using a deficiency lacking the lncRNA complemented the Mimic phenotype. In the future, if one were to pursue this work, they would need to create CRISPR mutants to ensure precise deletion of the lncRNA locus. Additionally, using FISH to determine where each lncRNA localizes would be useful.

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Caitlin Pozmanter

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Education

Johns Hopkins University — 2014-present
Baltimore, MD
Ph.D. Cellular, Molecular, and Developmental Biology and Biophysics
Certificate in Science Outreach

McDaniel College — 2008-2012
Westminster, MD
BA Biology, specialization in molecular biology
Cum Laude with honors in biology

Research Experience

PhD Candidate (Johns Hopkins) — 2014-present
Principle Investigator: Mark Van Doren Ph.D
Topic: Characterization of Tudor5-prime, a tudor domain protein with putative function in germ cell RNA granules.

Research Technician (Stony Brook University) — 2012-2014
Principle Investigator: Steven Glynn Ph.D
Topic: Mitochondrial AAA proteins

- Studied the structure and function of mitochondrial AAA proteins through x-ray crystallography, and various biochemical assays
- Trained graduate students and was a mentor for undergraduates and a masters student

Undergraduate Research Student (McDaniel) — 2009-2011
Principle Investigator: Susan Parrish Ph.D
Topic: mRNA decapping enzymes in *Dictyostelium discoideum*

- Cloning and biochemical characterization of *D. discoideum* DDB_G0278957

Genomics Education Partnership Research (McDaniel) — 2011
Principle Investigator: Susan Parrish Ph.D
Topic: Annotation of regions of the *Drosophila erecta and Drosophila mojavensis 3L chromosomes*

- For the lab component of the Genomics course we participated in the genomics education partnership (GEP) Specifically I annotated segments of the *D. erecta* and *D. mojavensis* 3L chromosomes

Teaching Experience

Adjunct Lecturer (McDaniel College) 2016-present
Designed an upper level course on RNA biology
Taught the lecture portion of Principles of Biology

Internship Mentor (Johns Hopkins) 2018-present
Have hosted two McDaniel students in my PhD lab for experiential Learning experiences in the form of biology research internships
-Harrison Curnutte: Jan term 2018, summer 2018
- Sydney Kelly: Summer-Fall 2019

Instructor (Johns Hopkins) 2018
Taught an intersession course titled "Introductory RNA seq analysis using R"

Teaching Assistant (Johns Hopkins) 2015-2016
Genetics lab and Developmental biology lab

Genomics Classroom Assistant (McDaniel) 2012
Assisted genomics students with their GEP annotation projects and tutored students prior to exams

Tutor for Student Academic Support Services (McDaniel) 2011-2012
Cell Biology, Organic Chemistry, Introduction to Chemistry, Basic Algebra, and Forensic Chemistry

Scientific Publications

Shekerah Primus, Caitlin Pozmanter, Kelly Baxter, and Mark Van Doren. (2019)"*tudor-domain containing protein 5-prime* promotes male sexual identity in the *Drosophila* germline and is repressed in females by *Sex lethal*" *Plos genetics*

Hong Zhou, Cale Whitworth, Caitlin Pozmanter, Megan C. Neville, Mark Van Doren (2018). "fruitless functions downstream of doublesex to promote sexual dimorphism of the gonad stem cell niche" Accepted Plos Genetics

Jing Xie , Matthew Wooten, Vuong Tran , Bi-Chang Chen , Caitlin Pozmanter , Christine Simbolon , Eric Betzig, Xin Chen (2015) "Histone H3 Threonine 3 Phosphorylation Regulates Asymmetric Histone Inheritance in the Drosophila Male Germline" Cell.

Leung, W, ...[Pozmanter, C,... Parrish, S],...Elgin, SCR (2015) "The Drosophila Muller F elements maintain a distinct set of genomic properties over 40 million years of evolution." G3: GENES, GENOMEs, GENETICS. [Total 940 student co-authors, 74 faculty co-authors.]

Scientific Presentations

Caitlin Pozmanter*, Sydney Kelly, Harrison Curnutte, Mark Van Doren. 2021. Tudor5-like defines a novel germline granule that regulates distinct aspects of differentiation in *Drosophila*. *62th annual Drosophila research conference*. Virtual. Talk

Caitlin Pozmanter*, Sydney Kelly, Harrison Curnutte, Mark Van Doren. 2020. Tudor5-like defines a novel germline granule that regulates distinct aspects of differentiation in *Drosophila*. 2020 Germ Cell meeting CSHL. Virtual. Talk

Caitlin Pozmanter*, Sydney Kelly, Harrison Curnutte, Mark Van Doren. 2020. Tudor5-like promotes germline differentiation through post-transcriptional gene regulation and maternal RNA regulation. The Allied genetics Conference. Virtual. Poster

Sydney Kelly*, **Caitlin Pozmanter**, Mark Van Doren. 2019. Tudor-domain-containing protein 5-like post-transcriptionally regulates maternally deposited RNAs in *Drosophila*. *22nd Annual Undergraduate Research Symposium in the Chemical and Biological Sciences.* University of Maryland Baltimore County, MD. Poster

- Sydney Kelly (McDaniel Student) won 1st place in the Biochemistry and Molecular Biology category

Caitlin Pozmanter*, Shekerah Primus, Leif, Benner, Harrison Curnutte, Mark Van Doren. 2019. Tdrd5l promotes germline differentiation through post-transcriptional gene regulation in cytoplasmic RNA granules. *24th Annual Meeting of the RNA society.* Krakow, Poland. Poster

Caitlin Pozmanter*, Shekerah Primus, Harrison Curnutte, Mark Van Doren. 2019. Tdrd5p promotes germline differentiation through post-transcriptional gene regulation in cytoplasmic RNA granules. *60th annual Drosophila research conference.* Dallas, TX. Talk

Caitlin Pozmanter*, Shekerah Primus, Mark Van Doren. 2018. TDRD5P promotes germline differentiation through post-transcriptional regulation in cytoplasmic RNA granules. *59th annual Drosophila research conference.* Philadelphia, PA. Talk

Binglin Chen*, **Caitlin Pozmanter**, Mark Van Doren. 2017. Functional analysis of sexually dimorphic long noncoding RNAs in the *Drosophila* germline. *20th Annual Undergraduate*

Research symposium in the Chemical and Biological Sciences. University of Maryland Baltimore County MD. Poster

- Binglin was an undergraduate I mentored. Her poster won 2nd place in the biochemical sciences category

Caitlin Pozmanter*, Shekerah Primus, Mark Van Doren. 2017. TDRD5P, a component of cytoplasmic processing bodies promotes male germline sexual identity. *58th annual Drosophila research conference.* San Diego CA. Talk

Caitlin Pozmanter*, Mark Van Doren. 2017 *tudor5-prime* regulates sex determination and germline differentiation in *Drosophila.* McDaniel College Biology seminar. Westminster MD. Invited Talk

Caitlin Pozmanter*, Shekerah Primus, and Mark Van Doren. 2016. The novel tudor-domain containing protein TDRD5P regulates male germline sexual identity. *57th annual Drosophila research conference*. Orlando FL. Poster

Anna Kokubu*, **Caitlin Pozmanter**, and Susan Parrish. 2014. Expression and purification of *Dictyostelium discoideum* DDB_G0278957 protein, a putative mRNA decapping enzyme. *17th Annual Undergraduate Research symposium in the Chemical and Biological Sciences. University of Maryland Baltimore County MD.* Poster

Hui Shi, Anthony J. Rampello*, **Caitlin Pozmanter***, Steven E. Glynn. 2014. Defining the Rules of Substrate Recognition by a Reengineered AAA+ Protease. 2014 Department of Biochemistry and Cell Biology Retreat. Stony Brook, NY. Poster

C. Pozmanter*, K. Moran, and S. Parrish. 2011. Cloning and Biochemical Characterization of *Dictyostelium discoideum* DDB_G0278957, a Putative mRNA Decapping Enzyme. *14th Annual Undergraduate Research symposium in the Chemical and Biological Sciences. University of Maryland Baltimore County, MD.* Poster

*denotes presenter

Awards, Honors, and Grants

RNA society travel fellowship	2019
Hopkins GRO travel grant	2019
National Science Foundation Graduate Research Fellow	2016-present
Victor Corces award for excellence in teaching (genetics)	2016
Cum Laude	2012
College Scholar	2012
Case Award for Excellence in Scholarly Research	2011
Who's Who in American Colleges and Universities	2011, 2012
Centennial Conference Winter Honor Roll	2010-2012
Member of Academic all America Swim Team	2010-2012

McDaniel College Phenomenal Women Nominee	2012
Dean's List: Honors	2011
Dean's List: Highest Honors	2008, 2012
Honors Program	2008-2012
Beta Beta Beta: *national biology honors society*	2009-2012
Gamma Sigma Epsilon: *national chemistry honors society*	2011-2012
Omicron Delta Kappa: *national leadership honors society*	2011-2012
Alumni Leadership Program for Seniors	2011-2012

Science Outreach, and Departmental service

Mentor for 1000 girls 1000 futures program	2019-present
Coordinator for CMDB 1st year big sib/little sib mentor program	2018-2019
Dept advocate at the Celebrate Life Sciences fair for Congress	2018
Thomas Hunt Morgan Award Committee	2016-present
Class representative to CMDB program administration	2014-present
Mentoring to Inspire Diversity in Science	2014-present
NSF Research Experience for undergrads mentor coordinator	2015-present

Professional Societies

Genetics Society of America	2016-present
RNA Society	2018-present